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CONTENTS

Cellular Reactions to Phthienoic Acid and Related Branched-Chain Acids. HAIDAR HUSSEINI AND SANFORD ELBERG 655

Recrudescence in Early Phthisis. A Study of Post-Mortem and Lobectomy Specimens. WALTER PAGEL 673

Observations on the Effect of Amithiosone (Tibione) in Selected Tuberculous Pulmonary Lesions. WILLIAM G. CHILDRESS, JANE W. NORMAN, ROY H. OTT, JR., AND DAVID M. SPAIN. 692

Fluorescence Microscopy in Examination of Smears for *Mycobacterium Tuberculosis*. MICHAEL M. WILSON 709

Antituberculous Activity in Mice of Triton A-20, a Nonionic Alkyl-Aryl Polyether Alcohol, Used Alone and in Combination with Dihydrostreptomycin. MORRIS SOLOTOROVSKY AND FRANCIS J. GREGORY 718

Enzymatic Reactions of *Mycobacterium Tuberculosis* and the Action of Streptomycin. H. J. CORPER AND VETILE D. JAMES 722

Tuberculosis and Sickle-Cell Anemia. WILLIAM WEISS AND S. O. WAIFE 735

Case Reports: Spontaneous Hemopneumothorax. A Review with a Report of Two Cases. DONALD A. FUSIA, JR. AND W. LEIGH COOK, JR 744

Notes:

Mycobacteria Resistant to Hydrazines of Isonicotinic Acid. WILLIAM STEENKEN, JR. *et al.*; MARGARET BUCK AND ROBERT J. SCHNITZER; FELIX PANSY *et al.*; GARDNER MIDDLEBROOK; WACLAW SZYBALSKI AND VERNON BRYSON; GLADYS HOBBY AND TULITA F. LENERT 754

Action of Streptomycin and Isonicotinic Acid Hydrazide on *M. Tuberculosis* Within Phagocytes. EMANUEL SUTER 775

Synergistic Action of Streptomycin and Isonicotinic Acid Hydrazide *in Vitro*. JAN ILAVSKY 777

Urease Activity in *Mycobacteriaceae*. JACQUES SINGER AND ERNA CYESKER 779

The Failure of Chick Embryo Extract to Accelerate the Growth of Tubercle Bacilli. SAMUEL ABRAMSON 783

American Trudeau Society: Report of the Eighth Pembine Therapy Conference, Michigan, Wisconsin, and Minnesota 786

Index of Authors 793

Index of Subjects 799

Index of Abstracts 804

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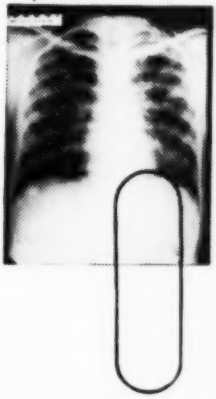

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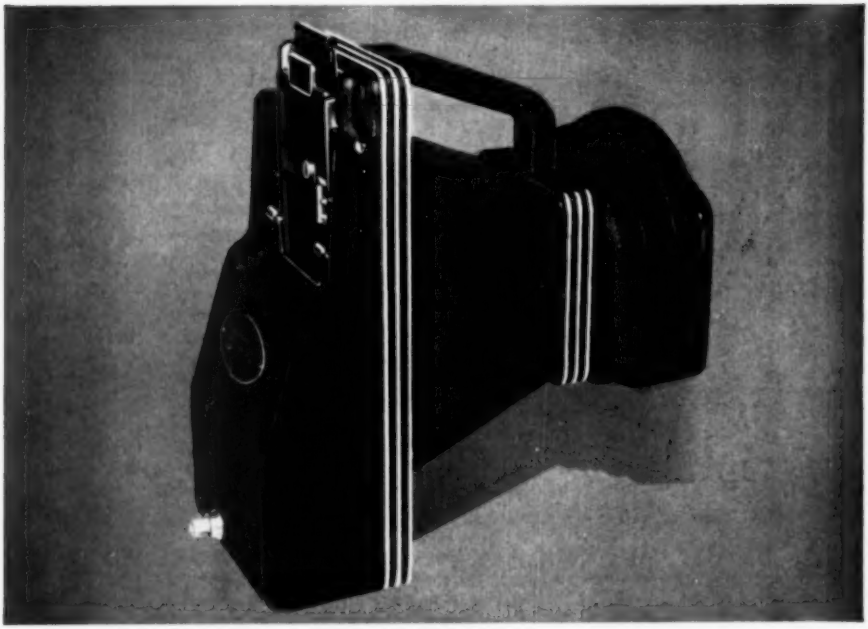

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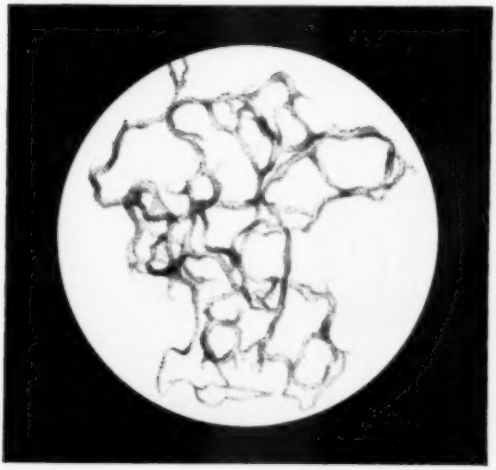

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ERRATUM

In Volume 65, No. 2, 1952, "Prognosis of Tuberculous Meningitis" a correction should be made on page 179 as follows:

Regimen I: Streptomycin intrathecally, 50 to 100 mg. *per kg.* daily
Regimen II: the same
Regimen III: Streptomycin 25 to 50 mg. *per kg.* intrathecally *daily* at start

should read:

Regimen I: Streptomycin intrathecally, 50 to 100 mg. *per injection.*
Regimen II: the same
Regimen III: Streptomycin intrathecally 25 to 50 mg. *per injection twice daily* at start.

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CELLULAR REACTIONS TO PHTHIENOIC ACID AND RELATED BRANCHED-CHAIN ACIDS[1]

HAIDAR HUSSEINI AND SANFORD ELBERG

(Received for publication January 10, 1952)

INTRODUCTION

Recent studies on a fraction of acids of the phthioic type isolated from *M. tuberculosis* revealed the presence of at least twelve components, ranging in chain length from C_{23} to C_{31} (1). Approximately one-fourth of the total appeared to be one compound and, after isolation in pure state, it was determined that it was an α-β unsaturated acid for which the name C_{27} phthienoic acid was proposed (1). The probable structural relations of the acid were indicated by the formula (2):

$$C_4H_9-(CH_2)15-\underset{\displaystyle CH_3}{\underset{|}{CH}}-CH_2-\underset{\displaystyle H}{\underset{|}{C}}=\underset{\displaystyle CH_3}{\underset{|}{C}}-CO_2H$$

The mixture of acids of the phthioic type also contained other acids of structure similar to C_{27} phthienoic acid but of different molecular weight. After hydrogenation, a saturated branched-chain acid, termed C_{29} phthianoic acid, was isolated (2). Correlative studies conducted on a sample of the original phthioic acid of Spielman and Anderson (3) showed that the latter also contained approximately 70 per cent α-β unsaturated acid.

In view of the cellular response to phthioic acid reported by Sabin (4), the ability of C_{27} phthienoic and C_{29} phthianoic acids to induce inflammatory reactions was investigated. For comparison, the cellular response to a series of synthetic fatty acids of varying chain length (C_{18} to C_{27}) containing a branching methyl group located in either the 2, 6, 10, 14, or 18 position was determined. Examination was also made of the response to straight-chain synthetic acids of the C_{24} and C_{26} type, the former a saturated, and the latter an unsaturated, acid. In this way the influence on cellular response of position of methyl side chain, unsaturation and saturation, and the influence of length of the basic chain in a series of acids having a branching methyl group in the 2 position could be ob-

[1] From the Department of Bacteriology, University of California, Berkeley, California.

served. In table 1 may be seen pertinent data on the acids which have been studied in this investigation.

The C_{27} phthienoic acid was more active in stimulating fibroblastic proliferation and epithelioid and giant cell formation than the saturated C_{29}-phthianoic acid or the sample of phthioic acid.

The effect of position of the methyl side chain was illustrated by the increased degree of the acute type of response as the substituent group moved from the sixth to the tenth carbon of the C_{24}-series followed by a decreasingly acute but increasingly proliferative response as the substituent moved from the tenth to the eighteenth carbon. The chronic-type response to a C_{22}-acid with a methyl group substituted at the second carbon, however, resembled the response induced by a C_{24}-acid with its substituent methyl group located on the eighteenth carbon.

The inhibition of linear leukocytic migration appeared to be directly related to the ability of the acids to induce the acute type of response for the C_{24}-series but this was not a general property for acids of other chain lengths. The inhibition of leukocytic migration could not be explained directly in terms of oxygen uptake by the leukocytes.

METHODS

Preparation of acids[2]: Cason and his colleagues have described the methods for the preparation of phthienoic and phthianoic acids (1, 2), the 2-methyloctadecanoic and docosanoic acids (5), 6- and 10-methyltetracosanoic acids (6), 14-methyltetracosanoic (7), and 18-methyltetracosanoic acid (8). n-Tetracosanoic acid was prepared by way of the 6-keto acid, following the general scheme used for synthesis of branched-chain acids. The corresponding aldehyde was used in a Doebner reaction for synthesis of 2-hexacosenoic acid. The details concerning these preparations will soon be published elsewhere.

Preparation of solutions for injection: The total amount of each acid needed for a series of injections was suspended in 5 ml. of physiologic saline and neutralized with N/10 NaOH. After saponification the total volume was adjusted to 10 ml. This suspension was then homogenized by repeated passage through needles of increasing gauge number until the solution was visibly homogeneous. It was then shaken mechanically until homogeneous by observation with a ten times magnification ocular of the microscope.

Each acid was studied in rabbits injected intraperitoneally. Series of rabbits were injected daily for ten days (*Schedule 1*, table 1), rested for twenty-four hours, and sacrificed by an intravenous injection of air. The cellular responses of the omentum of animals so treated were compared with the omental response of series of animals given the total amount of acid in one injection (*Schedule 2*, table 1), after which the animals were rested for ten days, and sacrificed as above. Response-to-dosage studies were performed in all cases and every dose of acid was studied in each of 3 animals. When phthienoic acid was used, additional studies were made of the cellular response one, twenty-four, and seventy-two hours and one, two, and four weeks after a single injection of the acid. A group of rabbits was injected for ten days with sterile saline to serve as a control.

Specimens for histopathologic examination were fixed in 10 per cent formalin. Paraffin sections were stained with Harris' hematoxylin and eosin and mounted in picolyte.

Leukocytic migration determinations: The basic method of Martin and co-workers (9) was employed in which the linear migration of blood leukocytes on clotted plasma was measured.

[2] We are greatly indebted to Dr. James Cason for supplying the acids used in this investigation.

A modification of the method for constructing the chambers was developed in order to prevent leakage of the blood during their final preparation. Slides and coverslips were

TABLE 1

FATTY ACIDS STUDIED FOR THE HISTOLOGIC AND LEUKOCYTIC MOBILITY RESPONSES OF RABBITS

FATTY ACID	FORMULA	SCHEDULE 1 TOTAL DOSE	SCHEDULE 2 SINGLE DOSE
		mg	mg
2-Methyloctadecanoic acid	$CH_3(CH_2)_{15}CHCOOH$ $\quad\quad\quad

was important in determining the speed at which the melted paraffin covered the filter paper. When the paraffin reached the bottom of the U, the cell was taken out and the paraffin was allowed to solidify, thus holding the cell together. The clothespin was removed, and the cell was held by a clean forceps at a 90° angle from its previous position. The paraffin was allowed to climb by capillary action and cover the arms of the U-shaped filter paper.

The main walls of the cell consist of a standard microscope slide and a No. 1 coverslip (24 by 60 mm.). The narrow lateral walls are provided by the U-shaped filter paper, leaving one open side to fill the cell with blood. The cells were kept in the deep freeze for further use.

Careful attention was paid to the details of cleaning glassware, silicone coating, and heparin titration for anticoagulant activity as described in the basic communication (9).

The activity of all acids was studied at 0.01 M, 0.001 M, 0.0005 M, and 0.00025 M final concentration in the acid-blood mixture.

The effect of the acids on the oxygen uptake of leukocytes: Two milliliters of the aspirated buffy coat of rabbit blood containing 18,650 cells per cu. mm. were deposited in a Warburg cup containing 0.2 ml. of 20 per cent NaOH in the well. To the side arm of the cup was added 0.32 ml. of a M/100 solution of the fatty acid under test. One control flask received saline in place of the acid. Comparable numbers of erythrocytes in saline were placed in another cup to serve as a second control, bearing in mind the possibility that oxygen uptake by erythrocytes in the slide cells was the governing factor in the movement of the leukocytes. After a ten-minute equilibration period, the manometers were closed and initial readings obtained. Then the contents of the side arms were tipped in, and the oxygen uptake was followed for 130 minutes at 37°C.

RESULTS

Omental Response

Phthienoic acid: Phthienoic acid was tolerated during single and multiple injections without evident signs of irritation. The animals maintained their weight and appetite. The omentum macroscopically was heavy, edematous, slightly hemorrhagic, and shredded. In animals receiving a single injection, the organ revealed small nodules visible to the naked eye. Microscopically, the blood vessels were congested, but the endothelial cells were still intact and no evidence of hemorrhage was present. There was a copious infiltration of eosinophilic cells, especially around the milk spots. Some fibrin deposits were also detected, and the tissue appeared to be granular. The main lesion was composed of epithelioid and epithelioid giant cells, the arrangement of which simulated a tubercle. Central necrosis was marked in animals which were given the single injection and rested. Epithelioid giant cells appeared to surround the necrotic center, while epithelioid cells surrounded the giant cells. Polymorphonuclear neutrophils and eosinophils were sparsely distributed among the epithelioid cells. Fibroblastic proliferation was evident, and there was a clear attempt on the part of the fibroblasts to encapsulate the lesion. Lymphocytes and plasma cells were localized at the periphery of the lesion (figure 1).

The results with a total of one milligram injected in ten equal amounts were similar to those described above, but the lesion was smaller in size and of milder intensity.

When it was ultimately determined that phthienoic acid induced the most proliferative type of response and produced a lesion similar to that seen in the

more nonspecific aspects of tuberculosis pathology, it was the acid of choice to follow the development of such lesions. Five milligrams were injected at one time intraperitoneally in rabbits which were then sacrificed after one, twenty-four, seventy-two hours and one, two, and four weeks.

After one hour, the omentum was normal in gross and microscopic appearance, with the exception of a slight congestion of the vessels and margination of the

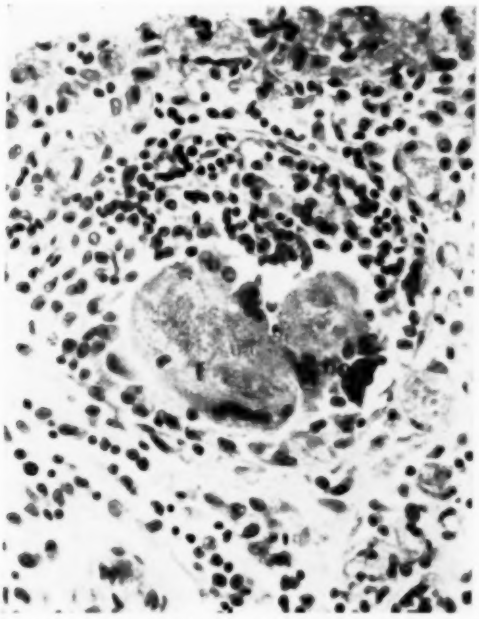

FIG. 1. The omental reaction of rabbits to phthienoic acid. Note central necrosis, giant cells, epithelioid cells, and fibroblasts encapsulating the lesion. (×500)

leukocytes; no diapedesis was detected, and neither leukocytic nor fluid exudation could be seen.

In twenty-four hours, the omentum was edematous and thickened, and slight hemorrhage had occurred. Microscopically, there was a copious polymorphonuclear exudation, with some mononuclear cells present. Diapedesis and margination were clearly seen. Fibrin deposits could be detected at different spots in the omentum. New capillary buds were noted.

In seventy-two hours the omental thickening had increased, but neither hemorrhage nor adhesions could be detected. Microscopically, there was a great decrease in the numbers of polymorphonuclear cells, the predominant cell at this time being mononuclear. Some epithelioid cells but no giant cells had de-

veloped. Fibroblastic proliferation was marked but this did not appear to encapsulate the area of inflammation.

Macroscopically, the results after one week were similar to those seen in seventy-two hours. Microscopically, the epithelioid cells increased in quantity, and the fibroblastic proliferation continued, but in these sections there was a clear attempt to encapsulate the inflamed area. No giant cell formation occurred. Some eosinophils could be discerned.

By two weeks the omentum had thickened and was studded with white nodules. There was a large nodule on the serosa of the intestine. Microscopically, the central part of the serosal nodule was composed of a necrotic center, karyorrhexis was evident, and nuclear debris was scattered over the necrotic center. Epithelioid and giant cells were seen around the necrotic center, and fibroblasts had completely encapsulated the lesion. Some eosinophils were interspersed between the epithelioid cells. The arrangement was clearly that of a tubercle.

The omentum after four weeks was normal, showing no thickening, hemorrhage, or nodular structures. Microscopically, some parts of the omentum were completely normal, while in other parts the repair processes were taking place. The fatty tissue was surrounded by proliferating fibroblasts and newly formed capillary buds. Fibrin deposits were clearly seen, and many long strands of fibrin had formed. In the center of the proliferating fibroblasts, the tissue appeared almost normal, with some fibrin deposits intermingled with the adipose tissue. A few mononuclear epithelioid cells were detectable on the inner side of the proliferating fibroblasts. The vascularity and the cell structure of the omentum were returning to normal condition. Thus, the reaction was like that induced by phthioic acid in the earlier studies of Sabin (4).

Phthianoic acid: Phthianoic acid produced gross changes in the omentum similar to those seen in response to phthienoic acid, but to a lesser degree. The blood vessels of the omentum were congested, but the endothelial cells were intact. Infiltration of polymorphonuclear cells and fibrin exudation were seen, but not to the same extent as in the case of animals injected with phthienoic acid. Epithelioid cells were the main cells of the lesion. They arranged themselves as in a tubercle. Lymphocytes surrounded the compact epithelioid cells. Fibroblastic proliferation was noted, but *there was no specific attempt to encapsulate the lesion.* Eosinophils could not be detected, as was the case with the previous acid. Mononuclear cells were diffusely scattered in the areolar structure (figure 2). Weight for weight, the unsaturated acid, namely, phthienoic acid, was the more active.

2-Hexacosenoic acid: The response to 2-hexacosenoic acid (figure 3) administered by both schedules was studied in order to determine: (*1*) the influence of a C_{26} unsaturated and unbranched acid on the general cellular response, and (*2*) whether eosinophils responded as in the case of phthienoic acid. Macroscopically, the omentum was thickened but intact, and showed no hemorrhage or adhesions. Microscopically, several lesions were observed on the omentum and peritoneum. Numerous giant cells with slitlike vacuoles were observed. Monocytes and a few polymorphonuclear cells were also present. These lesions were not surrounded

by fibroblastic proliferation, although proliferation of the fibroblasts was seen. The reaction was of the foreign-body giant cell type.

Comparison of substituted tetracosanoic acids with n-tetracosanoic acid: The response to the tetracosanoic series of acids in which a branching methyl group was substituted on either the 6, 10, 14, or 18 carbon atom in the fundamental chain was compared with the reaction to n-tetracosanoic acid by both schedules of injections.

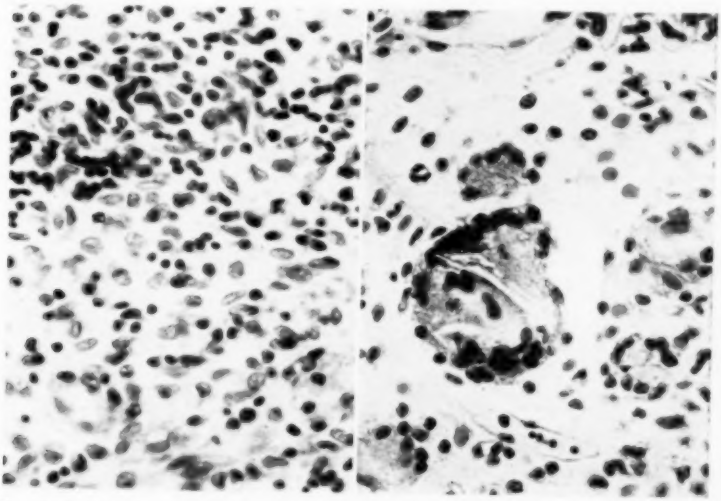

FIG. 2. (Left) Omental reaction of rabbits to phthianoic acid. Note compact arrangement of epithelioid cells. (×820)

FIG. 3 (Right) The omental reaction of rabbits to 2 hexacosenoic acid. Note presence of giant cells with a slit-like vacuole. The reaction is of the foreign-body reaction type. (×800)

n-Tetracosanoic acid: n-Tetracosanoic acid stimulated the formation of small nodular structures on the omentum and some edema. The characteristic cellular response to the injection of this acid was of the foreign-body type (figure 4). Numerous giant cells were apparent. Monocytes and a few polymorphonuclear cells were diffusely scattered in the areolar structure. The lesions were not surrounded by fibroblasts, and there was no attempt to encapsulate them; instead, the fibroblasts were sparsely scattered.

6-Methyltetracosanoic acid: In response to 6-methyltetracosanoic acid, neither adhesions nor hemorrhage were observed. A large nodule was found at the site of inoculation. The omentum was studded with small white nodules which simulated tuberculous tissue in their appearance. The cellular reaction (figure 5) was not an intense one; there was much less diffuse inflammation in the

omental areolar structure; and, in fact, there were some places where there was no exudation of cells at all. A few polymorphonuclear cells, plasma cells, and mononuclear cells were present in some places. The vascularity of the structure was not conspicuous. One nodular focus occurred in the lymphatic channel, probably on the surface of the wall of the lymphatic channel; while a second was a larger nodule, composed of large mononuclear cells and one giant cell. The giant cell was young and could have been either of the foreign-body type or of the Langhans' giant cell type. A few epithelioid cells were found in the pe-

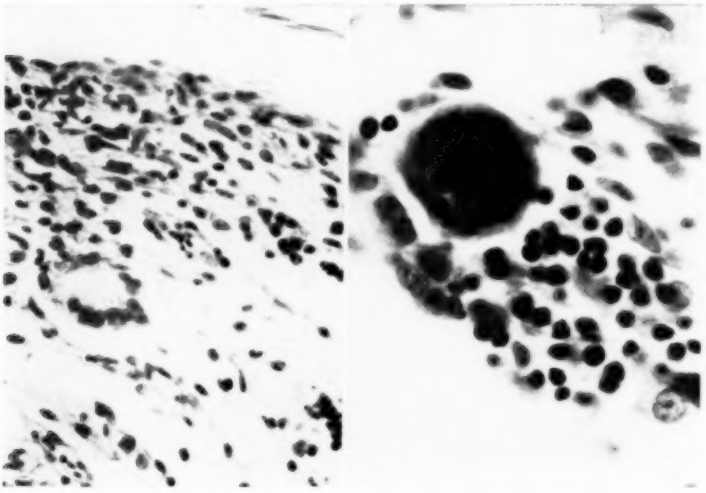

FIG. 4. (Left) Reaction of rabbit omentum to n-tetracosanoic acid. Note the giant cells in the section. The reaction is of the foreign-body type. (×800)

FIG. 5. (Right) The omental response reaction of rabbits to 6-methyltetracosanoic acid. Note epithelioid and giant cell reaction. (×1,370)

riphery of the nodule. Proliferation of the connective tissue, especially the fibroblasts, was evident. No necrosis was seen, and the reaction was of the foreign-body type.

Animals treated according to *Schedule 2* responded very similarly to those of *Schedule 1*, except for the increased fibroblastic proliferation and a clear attempt to encapsulate the lesion.

10-Methyltetracosanoic acid: The 10-methyltetracosanoic acid induced gross changes similar in appearance to the preceding analogue. The areolar omental tissue showed irregular, diffuse thickening, the inflammation being localized at one point, which consisted of a mixed inflammatory exudate. Polymorphonuclear cell infiltration, many large mononuclear cells, and the proliferation of epithelioid cells were observed. A few lymphocytes and plasma cells were noted at the

periphery of the lesion. Fibroblastic proliferation was prominent, particularly at the margin of the lesion, indicating an attempt to localize the irritant. Many capillary buds were evident. There was considerable karyorrhexis and possibly a little karyolysis. Some eosinophilic bodies were clearly seen. Giant cells were not present. This lesion was of the granulomatous type and resembled the tubercle in many respects (figure 6)

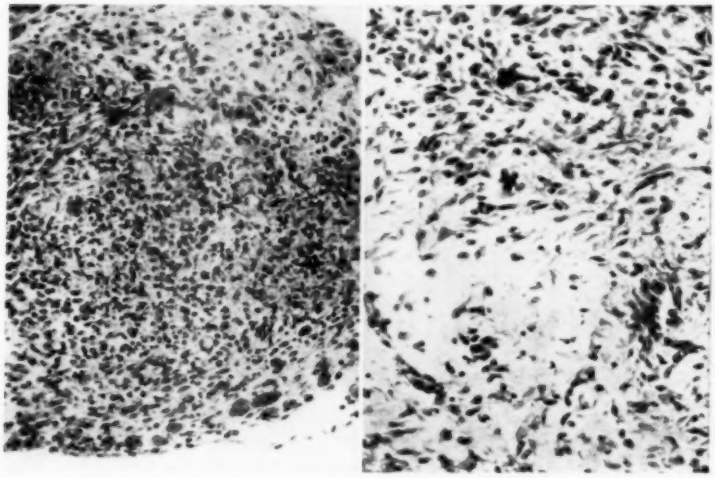

FIG. 6. (Left) The omental reaction to 10-methyltetracosanoic acid. Note central necrosis, with nuclear debris of polymorphonuclear cells; epithelioid cells are present. Fibroblastic proliferation is clear. (×282)

FIG. 7. (Right) The reaction of rabbit omentum to 14-methyltetracosanoic acid. Note fibroblastic proliferation and development of fibroblasts to fibrocytes. The reaction could be described as a fibrocytic nodule. (×332)

The main difference between the rabbits treated in accordance with *Schedules 1 and 2* was in the greater proliferation of the fibroblasts which tightly encapsulate the lesion in the latter case. There were approximately 4 or 5 cell layers of fibroblasts surrounding the necrotic center and completely walling it off, while the encapsulation of the lesion of rabbits treated in accordance with *Schedule 1* was not complete. Monocytes in various mitotic stages were seen.

14-Methyltetracosanoic acid: On *Schedule 1*, 14-methyltetracosanoic acid failed to produce grossly visible nodules but did so under *Schedule 2*. Slight thickening of the omentum was observed but no hemorrhage. Infiltration of leukocytes was prominent. Though a few leukocytes were present following *Schedule 1*, many more of them were evident following *Schedule 2*. Nuclear debris resulting from karyorrhexis was observed. This acid incited fibroblastic proliferation, and the lesion could be described as a small fibrous nodule (figure 7). The fibroblasts

were in no definite arrangement when *Schedule 1* was used; but with *Schedule 2*, the encapsulation of the necrotic center was very clear, surrounded completely by 5 to 7 layers of proliferating fibroblasts. Some fibroblasts developed to fibrocytes. The vascularity was increased, due to newly formed capillaries. The cellular response elicited by this acid closely resembled that of 10-methyltetracosanoic acid.

18-Methyltetracosanoic acid: The series of animals injected with 18-methyltetracosanoic acid yielded a chronic type of reaction. Plasma cells were the most prominent type in the reaction (figure 8). Some focal localization of plasma cells, especially around the vessels, occurred but many plasma cells were found away from blood vessels. Lymphocytic infiltration was present but to a small degree.

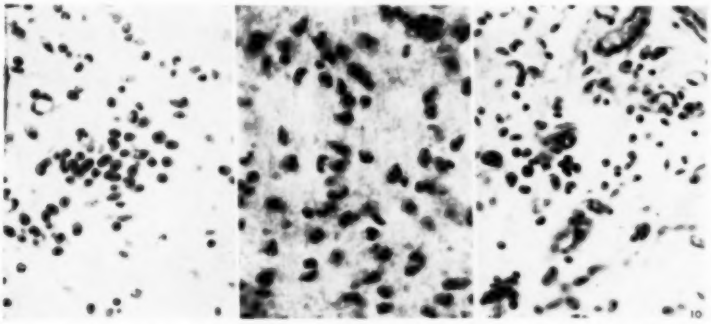

FIG. 8. Reaction of rabbit's omentum to 18-methyltetracosanoic acid. Note the focus of inflammation which is composed of plasma cells. The reaction is of the chronic type. (×820)

FIG. 9. The reaction of rabbits to 2-methyloctadecanoic acid. Note copious infiltration of leukocytes, erythrocytes, and monocytes. The reaction is of the acute inflammation type. (×1,300)

FIG. 10. The reaction of rabbits to 2-methyldocosanoic acid. Note the presence of the plasma cell. The reaction is of the chronic type. (×805)

A few polymorphonuclear cells were detected. Monocytes and mononuclear macrophages were not observed. Epithelioid and giant cell formation was absent. There was no fibroblastic development. The vascularity of the omentum was normal, and the areolar structure was intact.

The cellular response induced by this acid was very similar to that of 2-methyldocosanoic acid (figure 10) although the acids differ in both molecular weight and position of the methyl group.

2-Methyloctadecanoic acid: 2-Methyloctadecanoic acid produced gross hemorrhage and edema in the omentum. Microscopically, a marked polymorphonuclear neutrophilic infiltration occurred (figure 9). Some mononuclear cells and fibroblasts were seen. Clumped cells, the nature of which could not be determined, were noted among the mononuclear cells. Macrophages were present but were very scarce. In some portions of the section the omental cells had undergone

hyalinization. The vascularity of the structure was obscured, and no intact blood vessels were seen. Erythrocytes were diffusely scattered in the omentum, indicating severe hemorrhage. Nuclear debris of blue-staining fragments due to the karyorrhexis of polymorphonuclear cells was present all over the omentum.

The differences in responses to high and low doses were of degree rather than kind. The main difference between the animals treated in accordance with *Schedules 1 and 2* was that, following *Schedule 2*, the process of repair had started after ten days, and proliferation of fibroblasts and capillary buds were observed.

2-Methyldocosanoic acid: The most irritating substance of the series was 2-methyldocosanoic acid. Rabbit No. 102A lost some weight at the start of the injections, but as the injections were carried on it improved. Rabbit No. 102B died after receiving eight injections. The toxic effect of the acid was indicated after the injection, at which time the rabbits became uneasy; and, when returned to their cages, moved restlessly for a few minutes and then quieted down. Microscopically, loose, highly vascular areolar structures were observed, in which there was a diffuse infiltration of leukocytes of various types. Although the predominant cells were of the plasma type, large mononuclear cells were also seen together with a smaller number of lymphocytes. No nodule formation or local accumulation of cells was evident. The cellular reaction following *Schedules 1 and 2* was very similar, and no essential difference was noticed (figure 10).

Leukocytic Linear Migration

The rate of migration of leukocytes in general was remarkably uniform during the first ten hours. After twelve hours, the rate of linear migration had become distinctly slower. Even at that time, however, the leukocytes still appeared normal according to other criteria, such as random movement and motion of intracellular granules.

When leukocytes were placed in contact with phthienoic and phthianoic acid, their subsequent migration was inhibited. The degree of inhibitory effect depended on the ratio of acid concentration to phagocytes. The leukocytic response to 0.01 M acids is shown in figure 11. Phthienoic acid, when diluted to a final concentration of 0.00025 molar, was still slightly effective, while at the same concentration phthianoic acid had lost its inhibitive power.

Under the same experimental conditions, the effect of the synthetic fatty acids seemed to be less than that of the natural substances. Only 10-methyltetracosanoic acid affected the cells in a way comparable with phthienoic and phthianoic acids. The other acids, however, suppressed the migration of the leukocytes at the low dilution (0.01 M), but at higher dilutions they were almost inactive. The suppressive effect of these acids assumed somewhat the following order: 10-methyltetracosanoic acid > 2-methyldocosanoic acid > 2-methyloctadecanoic acid > 6-methyltetracosanoic acid > normal tetracosanoic acid > 18-methyltetracosanoic acid. The results in all graphs represent the arithmetic mean of five readings. The variation between the different readings was 0.2 to 0.3 mm., but the inhibitive power of these acids followed the same order in each

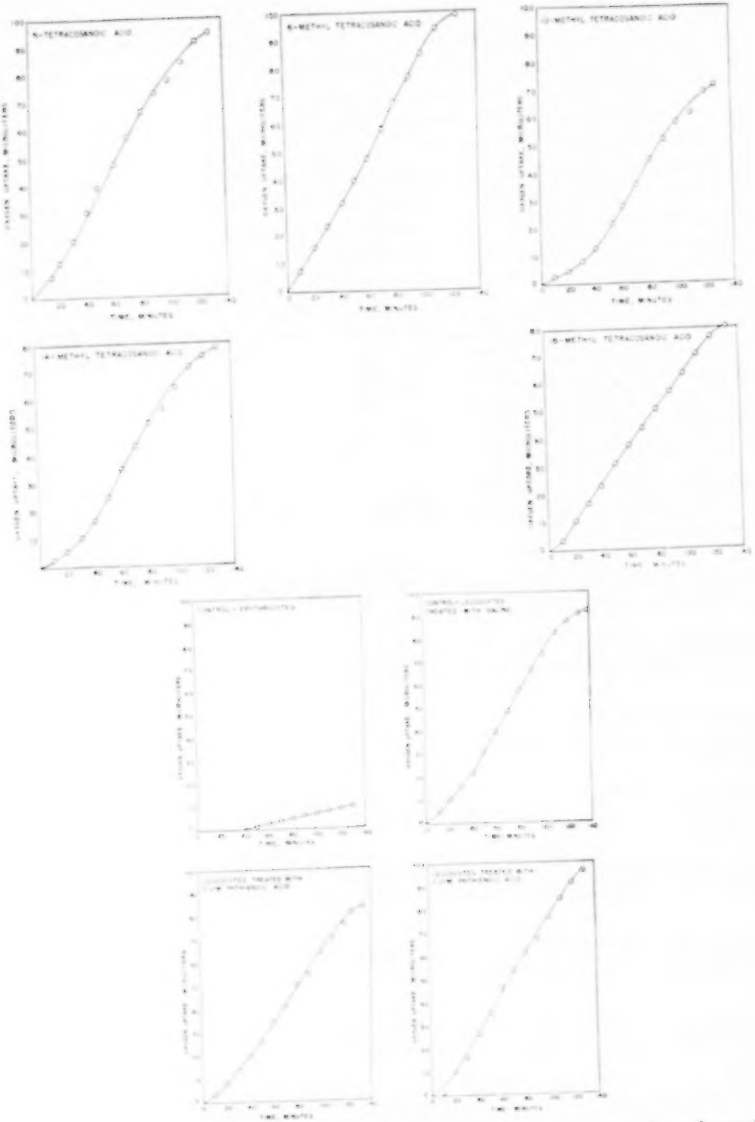

FIG. 11. Rate of leukocytic migration on plasma clot of rabbit polymorphonuclear cells in response to 0.01 M acids.

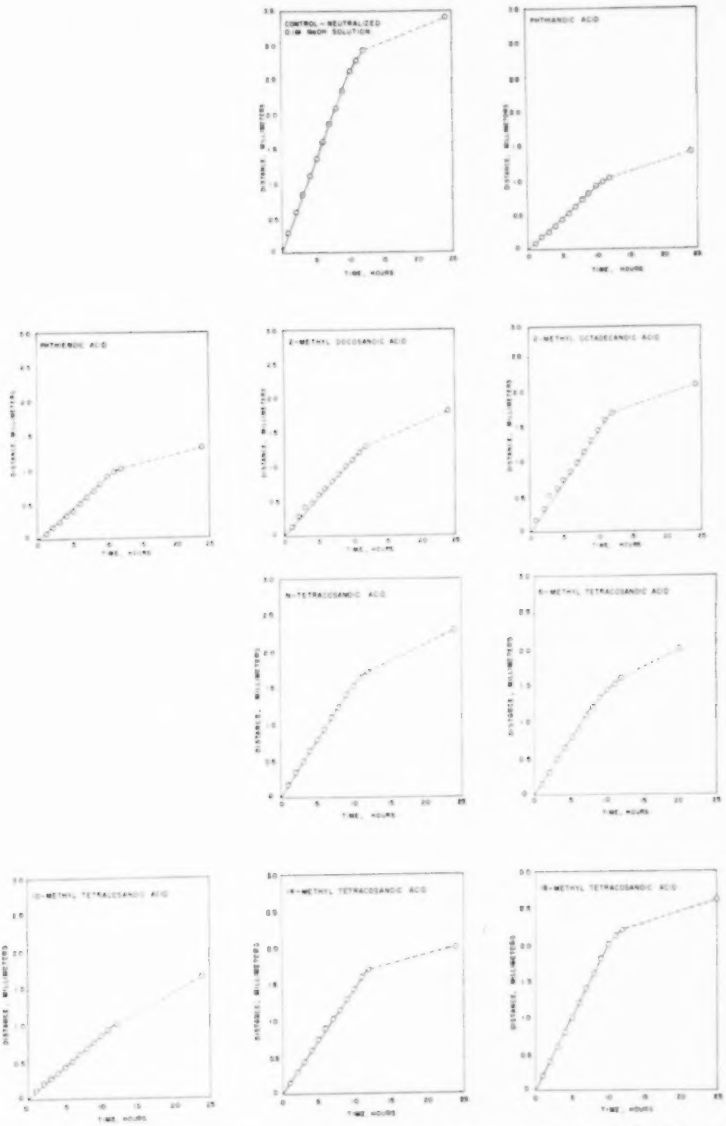

FIG. 12. Influence of branched-chain fatty acid on oxygen uptake of rabbit leukocytes treated with 0.01 M acids.

reading. The results indicated some correlation between the inhibitory power exercised by the acid and the acute nature of the cellular responses to these acids. However, this relation did not hold true for 2-methyloctadecanoic acid, 2-methyldocosanoic acid, and 18-methyltetracosanoic acid. While 18-methyltetracosanoic and 2-methyldocosanoic acid stimulated a very similar cellular response, their inhibitory power was markedly different. On the other hand, 2-methyloctadecanoic acid induced an acute type of response and demonstrated inhibitive power similar to that of 6-methyltetracosanoic acid, yet the latter acid induced a quite different cellular response. Another interesting observation was that agglutination of the leukocytes was evident with phthienoic acid, phthianoic acid, 2-methyldocosanoic acid, and 10-methyltetracosanoic acid. The other acids did not show this phenomenon.

The acids were entirely without effect on oxygen uptake of blood leukocytes, as measured by the Warburg technique (figure 12).

DISCUSSION

Phthienoic acid is strikingly active in stimulating the production of lesions which have much resemblance to some of those seen in tuberculosis, although the similarity has to do chiefly with the nonspecific aspects of tuberculosis pathology (10). Moreover, the experiments indicated that this acid was much more active than either its saturated analogue, phthianoic acid, or the tuberculolipids studied by Sabin (4). Nine hundred and sixty milligrams were used to produce a tubercular reaction in the case of the phosphatide A3 fraction (4) whose active ingredient was phthioic acid, while 1 mg. of phthienoic acid injected intraperitoneally was sufficient to incite the formation of epithelioid and epithelioid giant cells. Forty-five milligrams of phthianoic acid were needed to produce a lesion comparable to that of phthienoic acid. Since 1 mg. of phthienoic acid was assumedly not localized at any one certain focus but was free to spread throughout the peritoneal cavity, it may be assumed that the actual amount needed to incite epithelioid formation at any one site was much smaller. This is borne out by the fact that, with this small dose, the omentum was enlarged at certain points which were the sites of granulomatous lesions while other portions of the same omentum were histologically normal. This suggested that epithelioid and giant cell formation developed at the sites where the acid came in contact with the omentum and other membranes in the vicinity. Other interesting differences in the responses to phthioic (4), phthianoic acid, and phthienoic acid were the increased infiltration of the polymorphonuclear eosinophils and the greater proliferation of the fibroblasts induced by phthienoic acid.

Further chemical studies are needed to determine whether or not phthienoic acid is formed during the isolation procedure by dehydration of a beta-hydroxy acid originally present in the bacterial cell. This possibility is indicated by the presence of an hydroxyl group in mycolic acid (11) and it is being investigated in conjunction with Cason and co-workers.

The results of the tetracosanoic acid series indicated the importance of the position of the methyl group on the response. With n-tetracosanoic acid, the

reaction was proliferative, the main components of which were the foreign-body type giant cell, a few epithelioid cells, and very slight proliferation of fibroblasts; however, these elements did not assume an arrangement like that of a tubercle. With a methyl group substituted at position 6, the acid induced a more acute type of irritation; a lymphatic nodule was produced with a centrally located young giant cell, which was surrounded by some epithelioid cells and proliferating fibroblasts. Necrosis was insignificant, and plasma and lymphocytic cells were also present. Moving the methyl group to position 10 intensified the acute irritating power. Although the lesion was of the granulomatous type, it had a necrotic center composed of leukocytic debris arising from karyorrhexis and karyolysis of these cells. Epithelioid cells completely surrounded this necrotic center. Monocytes were seen at different mitotic stages, especially in the metaphase. The irritating power of 10-methyltetracosanoic acid was nearest to that of phthienoic acid, which suggested that a methyl group at position 10 or in its proximity might be found in phthienoic acid or acids of the phthioic type. The results in the present studies with the 10-methyl acid were similar to those obtained for it by Gerstl and Tennant (12). 14-Methyltetracosanoic acid induced less acute responses than were obtained with 10-methyltetracosanoic acid. The cellular response to the 14-methyl acid differed from that to the 10-methyltetracosanoic acid in the ability of the former to induce greater proliferation of fibroblasts and maturation of some of the fibroblasts to fibrocytes. At position 18, the methyl group was not as effective in this regard. The reaction was of the chronic type, the plasma cell being the main infiltrating cell type. Acute irritating activity seemed to increase as the methyl group substitution approached position 10, then began to decline when the methyl groups were substituted further from the carboxyl group.

Infiltration of leukocytes followed the initial injection with each of these fatty acids; however, leukocytic infiltration was not a feature of subsequent doses. The reaction to subsequent doses was more proliferative in type, wherein new monocytes developed along with epithelioid and giant cells. This phenomenon was not observed with 2-methyloctadecanoic acid which seemed to call forth the leukocytes after each injection, resulting in a sustained severe acute reaction. 2-Methyldocosanoic acid behaved like the other acids, the inflammation induced by its injection being very similar to that produced by 18-methyltetracosanoic acid. There was no obvious relation between the appearance of eosinophils and simple unsaturation.

The importance of molecular weight and of the number of carbon atoms was also clear, as pointed out earlier by others. Methyl groups were the only substituted groups employed because most natural substances have a methyl group substitution, and because of the analogy with tuberculostearic acid which was demonstrated to be 10-methyloctadecanoic acid.

The results of studies on leukocytic migration indicated that phthienoic acid had the most inhibiting power on leukocytic migration as compared with other fatty acids which were studied, and there seemed to be a correlation between irritating potency and inhibitive power. Phthienoic acid retained its inhibitive

capacity in 0.00075 mg. amounts, 10-methyltetracosanoic acid and phthianoic acid retained it in 0.0015 mg. amounts, and the others lost their inhibiting power almost completely in amounts of 0.003 mg.

The tantalizing question then arises whether phthienoic acid plays a role in the virulence of *M. tuberculosis*. If the correlation between virulence and the inhibition of migration of leukocytes is significant, as believed by previous workers (9, 13), then the results of the present studies suggest that phthienoic acid is one factor in the quality of virulence of these organisms. The chemical analysis of avirulent strains and their content of phthienoic acid will be important in evaluating this, a study which is in progress. It has been shown in this connection by Choucroun and co-workers (14) and others that the difference in inhibitive capacity of virulent and avirulent cells was quantitative; both types of cells were active, but fewer virulent cells were required for inhibition.

It is recognized that lesions similar to some of those described herein can be produced by a considerable variety of insoluble foreign particles in finely divided form and, furthermore, that comparisons of the activities of the various acids are difficult to represent in a limited number of illustrations. An effort to control these difficulties was made, however, by the examination of large numbers of sections of different organs. The evaluation of the differences of response was based, therefore, on the degree of complexity of the response. It is desired to focus attention principally on the quantitative aspects of the comparison between the activities of the acids used in the present study and the tuberculolipids studied previously (4).

SUMMARY

An ω-3 unsaturated acid of the phthioic type, designated as phthienoic acid, and its saturated analogue, phthianoic acid, were studied for their biologic activity.

The cellular response of rabbits to phthienoic acid and phthianoic acid consisted of epithelioid cells, giant cells, and tubercles. It was demonstrated, however, that phthienoic acid was quantitatively more active than its saturated analogue.

Phthienoic acid inhibited the migration of leukocytes on plasma clot in a manner comparable with that reported for virulent *M. tuberculosis* on the migration of leukocytes obtained from a susceptible host. Phthianoic acid was less inhibitory than phthienoic acid.

Synthetic methyl-substituted fatty acids were studied for their biologic activities. It was demonstrated by these studies that the position of the substituted methyl group played an important role in determining the type of the cellular response stimulated by these acids. When the methyl group was substituted up to the tenth carbon atom, the acute irritating power of the acid increased; when the group was substituted beyond the tenth carbon atom in the chain, the acid induced a more chronic type of response.

It was demonstrated that there was a limited correlation between the intensity of the cellular response and the effect of these acids on linear migration of

leukocytes. No correlation could be demonstrated between the effect of these acids on the migration of the leukocytes and on their oxygen uptake. The oxygen uptake of leukocytes treated with these acids was not affected.

SUMARIO

Reacciones Celulares al Acido Ftienoico y a los Acidos de Cadenas Ramificadas Afines

Un ácido insaturado α-β del tipo ftioico, denominado ácido ftienoico, y el análogo saturado del mismo, el ácido ftianoico, fueron estudiados con respecto a su actividad biológica.

La respuesta celular de los conejos al ácido ftienoico y al ácido ftianoico consistió en células epitelioideas, células gigantes y tubérculos. Además, quedó demostrado que el ácido ftienoico era cuantitativamente más activo que su análogo saturado.

El ácido ftienoico inhibió la migración de leucocitos sobre el coágulo de plasma en forma comparable a la descrita para el *M. tuberculosis* virulento con respecto a la migración de leucocitos procedentes de un huésped susceptible. El ácido ftianoico resultó menos inhibidor que el ftienoico.

Los ácidos grasos sintéticos con substitutos de metilo fueron estudiados en cuanto a su actividad biológica. Demostraron esos estudios que la posición del grupo metílico substituído desempeñaba un papel importante en la determinación de la forma de la respuesta celular excitada por dichos ácidos. Cuando la substitución del grupo metílico comprendía hasta el décimo átomo de carbono, aumentaba la facultad de irritación aguda del ácido; cuando la substitución traspasaba el décimo átomo de carbono de la cadena, el ácido evocaba una respuesta de forma más crónica.

Quedó demostrado que había una correlación limitada entre la intensidad de la respuesta celular y el efecto de dichos ácidos sobre la migración linear de leucocitos. No pudo descubrirse correlación entre el efecto de esos ácidos sobre la migración de los leucocitos y sobre la absorción de oxígeno por los mismos. No se afectó la absorción de oxígeno por los leucocitos tratados con dichos ácidos.

RESUME

Réactions cellulaires à l'acide phthiénoïque et aux acides apparentés à chaînes latérales ou secondaires

Un acide non saturé α-β du type phthioïque, dénommé acide phthiénoïque, et son analogue saturé, l'acide phthianoïque, ont été étudiés dans le but évaluer leur action biologique.

Chez le lapin, la réponse cellulaire à l'acide phthiénoïque et à l'acide phthianoïque a donné des cellules épithélioïdes, cellules géantes et des tubercules. Toutefois, il a été démontré que l'acide phthiénoïque possédait une activité quantitative plus élevée que son analogue saturé.

L'acide phthiénoïque inhibait la migration des leucocytes dans le caillot de plasma, d'une manière comparable à ce qui avait été observé pour le *M. tuberculosis* virulent sur la migration des leucocytes dont la source était un hôte sensible. L'acide phthianoïque était moins inhibiteur que l'acide phthiénoïque.

Des acides gras synthétiques, à groupe méthyle substitué, ont été étudiés pour déterminer leurs actions biologiques. Ces études ont démontré que la position du groupe méthyle substitué jouait un rôle important dans la détermination du type de réaction cellulaire provoquée par ces acides. Quand le groupe méthyle a été substitué jusqu'au dixième atome de carbone, le pouvoir irritant immédiat de l'acide augmentait; quand le groupe était substitué au delà du dixième atome de carbone de la chaîne, l'acide provoquait une réaction de caractère plus chronique.

Il a été démontré qu'il existait une corrélation limitée entre l'intensité de la réponse cellulaire et l'action de ces acides sur la migration linéaire des leucocytes. Aucune corrélation n'a pu être démontrée entre l'effet produit par ces acides sur la migration des leu-

cocytes et sur leur consommation d'oxygène. La consommation d'oxygène des leucocytes traités par ces acides n'était pas affectée.

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RECRUDESCENCE IN EARLY PHTHISIS[1]

A Study of Post-Mortem and Lobectomy Specimens

WALTER PAGEL

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Introduction

Any isolated source of bronchogenic spread of tubercle bacilli in the lung may be designated as an "early phthisical lesion." It need not necessarily be extensive and usually tends to cavitate or present itself as a fully developed cavity.

In "early phthisis," two main morbid anatomic features can be distinguished: (*1*) The recently acquired primary (or reinfection) focus becomes necrotic and forms a cavity, as is mainly observed in young adults ("primary cavity"); and (*2*) local recrudescence occurs in an old, usually calcified, focus followed by cavitation. This is either a primary focus or one which is additional to a quiescent primary lesion found elsewhere (postprimary focus).

The evidence for recrudescence has been fully investigated and documented as yet in only a small number of cases.

In the present paper, the role of recrudescence in early phthisis will be examined in 6 post-mortem and 6 lobectomy specimens; the latter allow a study of relevant changes at a much earlier stage.

Methods

The specimens were filled with formalin through the bronchial tree and, after three to eight days, were examined roentgenographically in various planes and were minutely dissected. Relevant parts of the lung and regional lymph nodes were examined histologically by multiple and, occasionally, by serial sections.

Case Reports

POST-MORTEM MATERIAL

Case 1

Clinical Findings

The patient was a man, aged 43 years, who gave a six months' history of gastric carcinoma, confirmed by laparotomy. On admission (December 1947), he had, in addition to gastric signs and symptoms, scattered rhonchi at the base of the right lung with prolonged expiration. The sputum was found to contain tubercle bacilli. A roentgenogram of the chest, obtained in November 1947, showed a group of calcified foci in the right mid-zone and the beginning of infiltration at the right apex. Another film in January 1948 showed very extensive exudative and cavitating disease throughout the right upper lobe and in the apex of the right lower lobe.

Post-Mortem Findings

At necropsy, performed on January 16, 1948 (No. 48/26), the body was that of a very cachectic man. A scirrhous carcinoma was found in the stomach at the pylorus.

[1] From the Central Middlesex Hospital, London, N. W. 10, England.

Lungs: The lower half of the right upper lobe showed several ragged cavities with serpiginous outline. At the apex, over which the pleura was particularly thick, were several firm, chalky-white foci, 0.5 cm. in diameter, with thick, gray capsules and surrounded by partly liquefying, porridge-like tissue (figure 1B). These foci showed up as densely opaque spots in the post-mortem roentgenogram and occasionally were surrounded with a less dense halo (figure 1A, arrow) which corresponded to the softened periphery of the nodules observed in the specimen. Six calcified nodules were in the right lower lobe. The largest nodule measured 1.2 by 0.2 cm. A group of small, calcified nodules were in the bifurcation lymph node.

Histologic examination of sections of the apex of the right upper lobe showed old caseous and calcified foci with elastic fibers preserved and forming an alveolar pattern. These areas

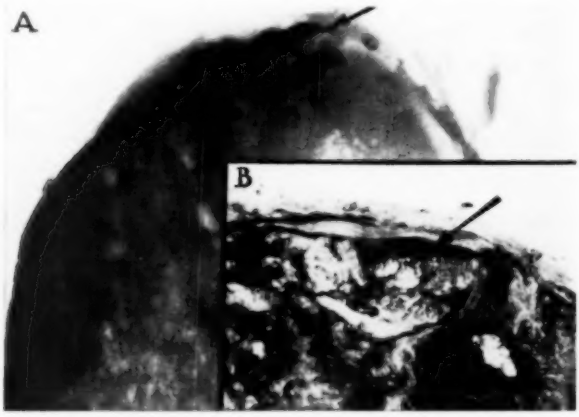

FIG. 1. Case 1. Male, 43 years old

A. Post-mortem roentgenogram of right apex; densely opaque spots with halos.

B. Right apex with chalky foci surrounded by porridge-like tissue ("pultaceous liquefaction").

of old tuberculosis were contiguous with liquefied, caseous tissue, in the substance of which large numbers of tubercle bacilli could be detected. Many of these were granular and stained pink with Giemsa.

Comment: A conspicuous feature was the presence of liquefaction in calcified foci, clearly demonstrated by the porridge-like consistence of the focus in which calcified debris seemed to be suspended. This was particularly noticeable in the post-mortem roentgenogram, in which the areas appeared as dense opacities with a less dense surrounding halo. Histologically, these areas showed disintegrating old caseous and calcified material suspended in fluid. In the solid parts, elastic fibers were preserved in an alveolar pattern. There was no appreciable cellular immigration into this area. Leukocytes and mononuclear cells, usually seen in cavitation, were absent. On the other hand, batches of acid-fast bacilli recalled the well-known picture of the early acellular stage of liquefaction, as described by Pagel (1, 2). Such pictures have been interpreted to support the view that liquefaction is actually initiated by bacillary multiplication.

No acid-fast organisms were found in those parts of the focus that consisted of dense fibrous and calcified material. In other areas, in addition to simple disintegration, swarms of leukocytes and mononuclear cells presented the typical picture of tuberculous cavity formation.

Softening with multiplication of tubercle bacilli occurring in a calcified lesion indicated recrudescence. Preservation of alveolar elastic fibers identifies the solid areas as patches of calcified lobular caseous pneumonia. This suggests recrudescence, rather than a false simulation thereof, by the healing of cavities with inspissation of contents when elastic fibers form irregular masses.

In the present case, recrudescence was limited to calcified postprimary foci; a firmly calcified primary complex was found in the right lower lobe. Recrudescence was probably due to malnutrition associated with gastric carcinoma.

Case 2

Clinical Findings

The patient was a man, aged 42, who, while a prisoner of war in Borneo for four years, had suffered from several tropical diseases. In August 1947, he was admitted with the diagnosis of subacute bacterial endocarditis. He had cough and some sputum which was positive for tubercle bacilli. A chest roentgenogram revealed a soft, rounded opacity, containing some pinhead-sized denser shadows, in the left upper zone (figure 2A). In tomographs, beginning cavitation was visible in the opaque area. Progressive illness with extensive hemothorax terminated in death within six months.

Post-Mortem Findings

At necropsy (No. 48/73) the classical picture of subacute bacterial endocarditis was found.

Lungs: The subapical area of the left upper lobe contained a round, encapsulated, caseous focus, 2.5 cm. in diameter (figure 2C). Within the focus several white nodules were found, each approximately 0.05 cm. in diameter and some calcified, which stood out in relief against the grayish-white, partly anthracotic background. The nodules appeared as opaque spots in a post-mortem roentgenogram (figure 2B). There was a small calcified nodule, 0.15 cm. in diameter, in the lower pole of the left upper lobe and a similar nodule in a regional bronchopulmonary lymph node (primary complex).

Histologic examination of the large, round focus showed it to have a thick, fibrous capsule which separated it from the surrounding collapsed lung. Inside the capsule, the focus was mainly one of old lobular, caseous pneumonia with patchy calcification and conspicuous areas of liquefaction which contained large numbers of granular and eosinophilic acid-fast bacilli. In some areas, a fibrous capsule surrounded disintegrating caseous material which was undergoing acellular liquefaction. Traces of elastic fibers in an alveolar pattern could still be seen in the liquefying areas (figures 2D, E, and F).

Comment: A prisoner of war with a six months' history of bacterial endocarditis was found to have tubercle bacilli in his sputum and a soft, round, infraclavicular opacity was noted in his chest roentgenogram.

This focus was identified at necropsy as a large, postprimary, circular, and caseous infiltrate in the left upper lobe. It was partly calcified, caseous pneumonia, partly liquefying, and contained in its substance small, calcified nodules which were strikingly visible in the post-mortem roentgenogram (figure 2B).

As in the previous case, acellular liquefaction, with very numerous tubercle bacilli, was seen in close contiguity with fibrosing and calcified caseous pneumonic tissue. This again indicates recrudescence of an old focus, probably after

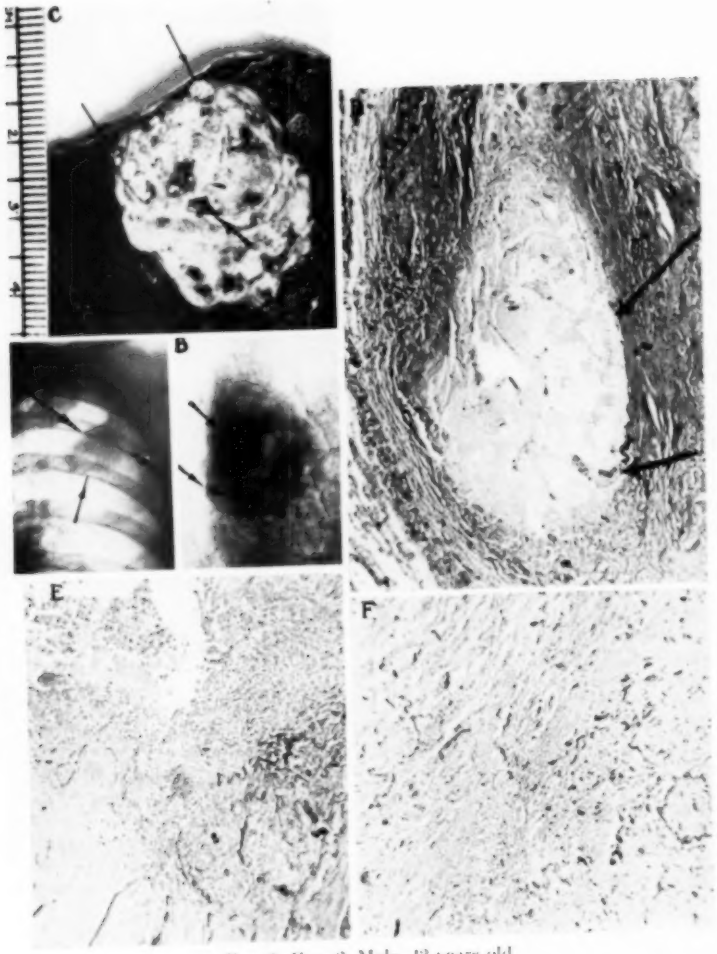

FIG. 2. Case 2. Male, 42 years old

A. Chest roentgenogram, August 1947. Large, circular focus in the left upper zone, with small calcified centers.

B. Post-mortem roentgenogram, with calcified spots and beginning softening at the center of the circular focus in left upper lobe.

C. Post mortem specimen of the circular focus, old caseous with calcified areas (thin arrow) and beginning cavitation at the center (thick arrow).

D. Area of acellular liquefaction showing remnants of elastic fibers. Weigert's elastic stain. (×40)

E and *F*. Liquefying areas with remnants of elastic fibers showing an alveolar pattern in the caseous parts. Weigert's elastic stain. (×80)

long or recurrent periods of quiescence, which is dependent on some factor favorable to bacillary multiplication; in this case, possibly malnutrition. Symptoms referable to the subacute bacterial endocarditis developed only after release from captivity, i.e., one year before death. Serial roentgenograms taken during the latter part of this period showed no changes in the appearance of the focus. It is, therefore, improbable that the endocarditis was principally responsible for the flare-up.

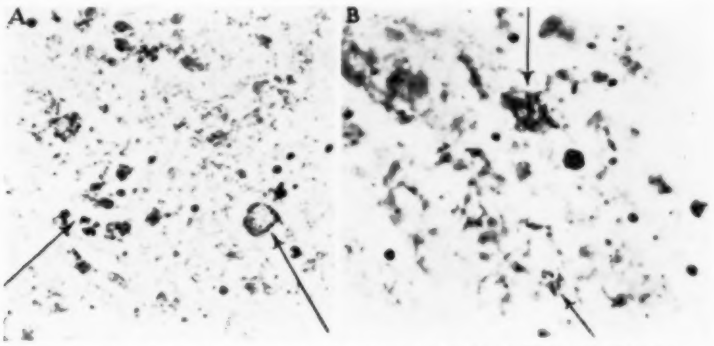

FIG. 3. Case 3. Central areas of old caseous foci with acellular liquefaction

A. Ziehl-Neelsen. (×600) Acid-fast elements mostly in a granular form.
B. Giemsa preparation. (×1,425) Granular eosinophilic bacilli.

Case 3

A male, aged 72, had one year's history of prostatic carcinoma.

Post-Mortem Findings

At post-mortem examination (No. 48/308), both upper lobes of the lungs, the left more than the right, contained several well-encapsulated, old caseous and calcareous foci. Some of these showed small areas of softening. No tuberculous cavity was found, but in both lower lobes near the base there were some areas of lobular, caseous pneumonia. The firmly calcified primary complex was found in the right lower lobe.

A post-mortem roentgenogram of the lungs showed, apart from the calcified primary complex, scattered calcified nodules in the upper lobes situated in some of the old caseous and calcareous foci described above.

Histologic examination of sections from various parts of both upper lobes showed a considerable number of old, lobular caseous pneumonic foci with thick, fibrotic capsules and masses of coal pigment therein. The peripheral parts of these foci consisted of compact old caseous tissue which contained cholesterol and other smaller crystals and much coal pigment. The central parts were in a state of acellular softening and disintegration and were replete with acid-fast bacilli. No such organisms were detected in foci without softening. In addition, numerous fresh caseous and liquefying areas were found which were full of acid-fast bacilli but without cavitation, i.e., a space with an independent caseous or tuberculous wall. The acid-fast elements found in the old caseous foci with central acellular softening were mostly granular and stained distinctly pink with Giemsa (figures 3A and B).

Comment: In an old man, dead of prostatic carcinoma, four groups of tuberculous lesions were found in the lungs: (*1*) the firmly calcified primary complex;

(*2*) a spread of well-encapsulated, old caseous-pneumonic, calcareous and small calcified nodules; (*3*) small areas of softening in some of the nodules described under (*2*); and (*4*) fresh caseous and liquefying changes due to bronchogenic spread.

Bacillary multiplication and softening in calcareous foci (recrudescence) supplied material for the bronchogenic ("phthisical") spread. At the time of death, this had caused some consolidation near the base. The changes suggest a continual development from a very old primary infection to the recent formation of sources of bacillary multiplication and bronchogenic spread. As in *Case 1*, the flare-up was associated with carcinomatosis.

Case 4

Clinical Findings

A man, aged 34, had pleurisy in 1939 and had subsequently been examined roentgenographically at periodic intervals. In 1944, he complained of lassitude but was well otherwise until Christmas, 1947, when he complained of a husky voice and was found to have tubercle bacilli in the sputum. In April 1948, he developed wheezing and cough and his temperature began to rise. On October 7, 1948 he was admitted in a severe status asthmaticus, and died four and one-half hours after admission.

Post-Mortem Findings

Post-mortem (No. 48/317) examination revealed acute emphysema of the lungs, with the pleura on the right side diffusely adherent. Mucopurulent and caseous tracheobronchitis was present, with formation of solid casts which were most conspicuous in the lower lobe bronchi.

The right upper lobe contained, in its dorsal part approximately 3 cm. below the apex, an oblong cavity 3 by 1¼ cm., with a fibrocaseous wall 2 to 2½ cm. thick. The bronchial outlet of the cavity was lined by a thick, caseous wall but could easily be followed into the right upper lobe bronchus.

Hard, calcified nodules were found to be intimately mixed with the fibrocaseous material at the dorsal end of the cavity. In a post-mortem roentgenogram, the right upper lobe showed the thick outlines of the cavity containing calcified debris (figure 4 inset). The calcified primary complex was seen near the interlobar fissure of the right upper lobe. The primary focus, 0.5 cm. in diameter, was situated at the lower end of the right upper lobe in its dorsal and subpleural aspect, and a calcified area 2 by 2 cm. was present in the right bifurcation lymph node. There were similar calcified areas in the right bronchopulmonary, tracheobronchial, and paratracheal groups. In the latter, the foci were calcified only in parts; the peripheral areas were still soft, chalky, and caseous. The left lung contained no fresh changes.

Histologic examination showed: (*1*) various forms and stages of tuberculous caseous tracheobronchitis; (*2*) the cavity wall thick, caseous, and, in some areas, covered with colonies of long, acid-fast rods. Marked fibrocellular proliferation was present in the periphery. (*3*) In the dorsal (still caseous) bottom parts of the cavity, an intimate mixture of old caseous tissue with calcified debris was found (figure 4).

Comment: This is the case of a man who probably acquired primary infection at the age of twenty-five. At the age of thirty he had had some symptoms of possible activity but developed sputum positive for *M. tuberculosis* and an overt illness only eight years after primary infection. For the last six months, he suffered from an ever-increasing, and finally suffocating, general caseous tracheobronchitis.

Necropsy revealed the primary complex which, although presumably nine years old, was not yet fully calcified but showed, at the end of a chain of calcified foci in one of the paratracheal lymph nodes, chalky and soft caseous material.

Calcified nodules scattered in the more dorsal part of a cavity in the right upper lobe must be regarded as the first postprimary manifestation which, in turn, was followed by formation of the cavity. The intimate mixture of the calcified material with the constituents of the cavity wall is evidence against an accidental involvement of quiescent calcified areas by a more recent and inde-

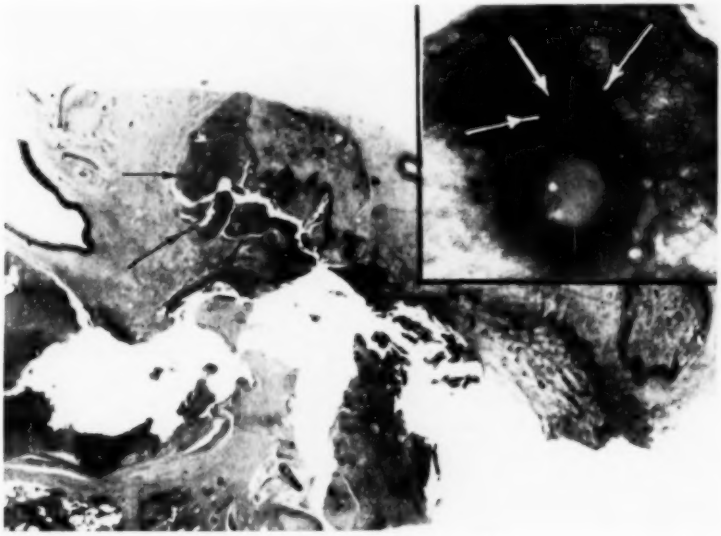

FIG. 4. Case 4. Male, 34 years old

Histologic survey (×6) of the cavity showing intimate mixture of calcified debris with caseous contents of the cavity wall. Inset: post-mortem roentgenogram of thick-walled cavity with calcified debris in caseous wall.

pendent fibrocaseous process. The pictures suggest recrudescence of a lesion which had partly healed by calcification, with subsequent slow local development of caseous and cavitating changes. It can be inferred that the calcified changes were not much younger than the primary complex, i.e., a little less than nine years old.

The case is suggestive of recrudescence of a calcified lesion which developed into a cavity.

Case 5

Clinical Findings

The patient was a male, aged 65 years, who had suffered from recurrent bronchitis for ten years. He had had pleurisy in 1940, when a chest roentgenogram showed "inactive

tuberculosis." In June 1942, a large infiltrative shadow was discovered in the lingula region. Most of it disappeared within two months but sputum became positive for *M. tuberculosis* in 1944, when some excavation seemed to develop in this region. Death occurred three years later as a result of cor pulmonale and bronchial asthma.

Post-Mortem Findings

At post-mortem examination (No. 50-47), a large, calcified tuberculoma, 2 by 2.5 cm., was found subapically and subpleurally in the right upper lobe (figure 5A). A small, calcified focus was found in a corresponding superior mediastinal lymph node, which was palpable in the specimen and showed up well in the post-mortem roentgenogram (figure 5B, inset). This focus was regarded as the lymph node complement of the tuberculoma in the right upper lobe (primary complex). There were multiple additional chalky foci, embedded in a fibrotic, subpleural, archlike scar which extended upwards into the right apex (figure 5B).

The left upper lobe contained a triangular, fibrotic area, 3 by 2.5 cm., with three round foci in the center. Two of these were dark gray in color and of porridge-like consistency, with beginning cavitation. The third was still solid and revealed several calcified centers which were seen most easily in the post-mortem roentgenogram (figure 5D). There was another similar fibrotic area with several small, chalky foci (0.8 to 0.5 cm. in diameter) with soft, almost liquefied centers. The histologic picture was one of lobular caseous pneumonia in a state of acellular liquefaction (figures 5E, F). Elastic debris was present in the central parts and elastic fibers were arranged in an alveolar pattern in the peripheral parts of the area. Groups of acinose-nodose foci could be seen scattered in both upper lobes. The histologic changes were substantially the same as those described in *Cases 1* and *2*.

Comment: An elderly man with recurrent bronchitis, bronchial asthma, and terminal cor pulmonale developed, within the eight years before death, infectious tuberculosis in the left mid-zone in the presence of calcified nodules in the right apex. Post-mortem roentgenography and dissection revealed a completely calcified primary tuberculoma in the right upper lobe, probably ten years old. In the left mid-zone, a fibrotic field contained several postprimary, partly calcified, partly cavitating foci. These presented the picture of porridge-like softening and formed the obvious source for groups of acinose-nodose foci and tubercle bacilli in the sputum, i.e., early bronchogenic phthisis.

SURGICAL MATERIAL

Case 6

A male, aged 16, was discovered in a mass roentgenographic survey to harbor a round focus in the right lower zone of the lung (June 1948). In subsequent roentgenograms, this appeared to increase in size and to cavitate near its lower pole. A roentgenogram taken in July 1950 revealed a calcified area in the focus and grossly calcified lymph nodes at the hilum.

Resected specimen: The right lower lobe, removed surgically on August 7, 1950, contained in its upper half a tennis-racket-shaped, lamellated focus (5 by 3 cm.) with a calcified core, 1 cm. in diameter, laterally in its upper half and a small cavity (1 by 0.3 cm.) in its lower half. The calcified core corresponds to the area seen in the roentgenogram of July 1950.

Histologic examination showed caseous tissue which varied in density from calcified, to old caseous, to liquefying areas. Calcified and liquefying parts were often found in close proximity to each other. The liquefying areas contained numerous acid-fast bacilli, polymorphonuclear and mononuclear cells, i.e., the classical picture of tuberculous liquefaction. Neither organisms nor cells were seen elsewhere in the focus.

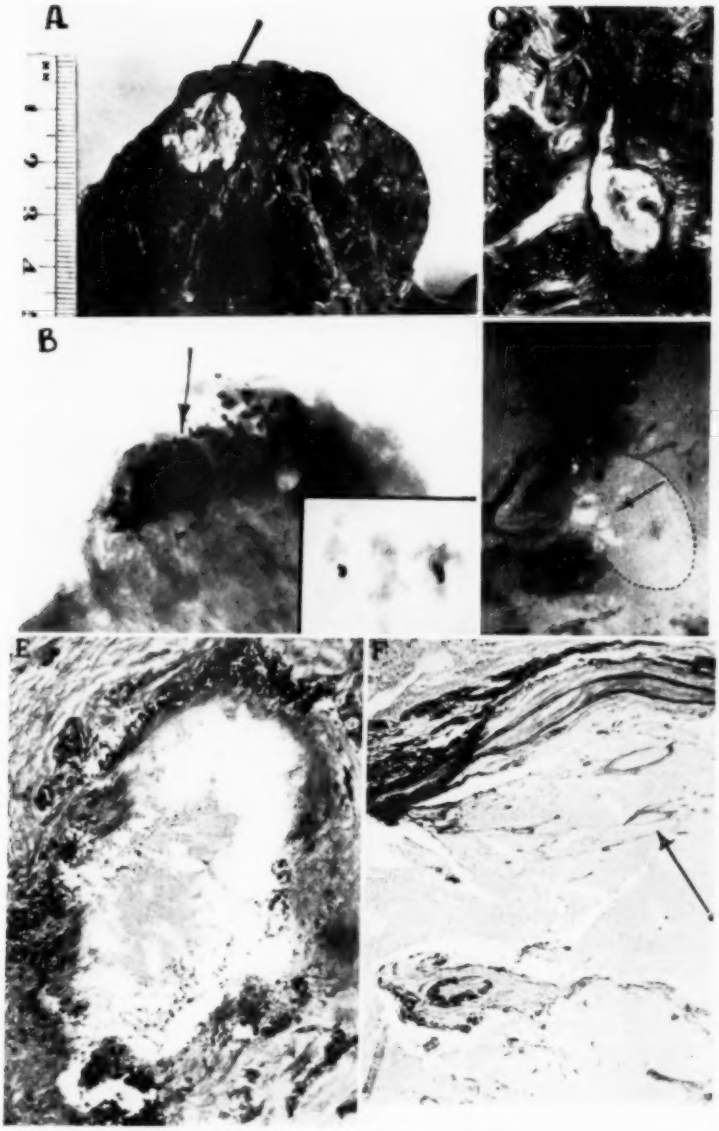

FIG. 5. Case 5. Male, 65 years old

A. Primary tuberculoma right apex.

B. Post-mortem roentgenogram. Primary calcified tuberculoma with multiple appositional calcified nodules. Inset: corresponding superior mediastinal lymph node with calcified focus.

C. Soft, chalky focus in the left upper lobe.

D. Post-mortem roentgenogram revealing calcified centers in the chalky focus.

E. Histologic survey of a chalky focus with acellular liquefaction. Hematoxylin-eosin. (×40)

F. Another similar area with preservation of elastic fibers in alveolar pattern. Van Gieson-elastica preparation. (×40)

No lymph node foci were found in the specimen, but the roentgenographic evidence of calcified hilar nodes seen in July 1950 could still be observed in postoperative roentgenograms.

Comment: This case involves a young adolescent who, in a series of roentgenograms and the lobectomy specimen, presented a lesion of the primary complex type. It consisted of a "tuberculoma" associated with calcified nodules in the hilar region. The evidence based on examination of a lobectomy specimen, even when supported by pre- and post-operative roentgenograms, was obviously more limited than information obtainable from post-mortem examination. Hence, the lesion was designated as one of the "primary complex type." The feature that was of particular interest in the present study was a large, solitary focus that was growing slowly by alternating caseation, calcification, and cavitation. In other words, it presented the hallmarks of recrudescence; while the lymph node component appeared to be completely healed.

Case 7

A female, aged 15, a family contact, in July 1948 was found to have a large, round focus in the right lower zone with a calcified nodule in the hilum nearby. On roentgenographic follow-up observation, the lung focus showed growth by direct extension within three months and eccentric breakdown within ten months (October 1948 and June 1949, respectively). A right lower lobe lobectomy was performed on July 8, 1949.

Resected specimen: The specimen contained a lamellated, caseous, partly calcified tuberculoma, 2.5 cm. in diameter, with a slit-like cavitation, 0.8 by 0.3 cm., in its upper and medial aspect. The lower and lateral third of this focus formed a chalky area, 1.3 by 0.7 cm., which in the roentgenogram of the specimen appeared, in its upper and lateral sector, as a firmly calcified nodule, 2 mm. in diameter. In histologic sections, this nodule was recognizable as a densely calcified area. It had no capsule but was marked out by its dense calcification against a less calcified, chalky-caseous bed. Traces of elastic fibers in alveolar pattern were found in all areas of the tuberculoma except those undergoing liquefaction. Two calcified regional lymph nodes, 0.4 by 0.2 cm. each, were also seen. The lobe, especially in the vicinity of the tuberculoma, was interspersed with small tubercles due to bronchogenic aspiration.

Comment: This is a further example of a solitary lesion of the primary complex type. In the pulmonary component, a densely calcified nucleus was revealed in the roentgenogram of the specimen, while the specimen itself showed a chalky area much larger in extent, with a slit-like cavity. This association of changes again provided evidence of slow growth with alternating calcification and cavitation, i.e., of recrudescence.

Case 8

A male, aged 28, whose chest roentgenogram had shown nothing abnormal in 1946, in September 1948 was found to have a large, rounded infiltrate in the left mid-zone of the lung.

Resected specimen: The lesion was removed by left upper lobectomy in March 1949 and proved to be a tuberculoma, 3.5 cm. in diameter, with a small, central cavitation in its upper half. A regional bronchopulmonary lymph node, 1.0 by 0.5 cm., was caseous. While roentgenograms and full dissection of the specimen disclosed no calcification elsewhere, the round infiltrate itself showed old caseous pneumonic tissue with a calcified fringe in the lateral sector of its lower half, as was also seen in a roentgenogram of the specimen. In the latter there was no demarcation between the calcified and caseous parts of the focus.

Comment: This case is an example of a tuberculoma, at the most of three years' standing, of the primary complex type in an adult. The feature of interest for the present series was the coexistence of marginal calcification with central cavitation.

Case 9

A female, aged 14, who had been under clinical supervision as a tuberculous "contact" for two years, in December 1948 developed a rounded infiltration in the right lower lung with swelling and patchy calcification of the hilar region. Roentgenographically (February 1949), the infiltrate was seen to be cavitating.

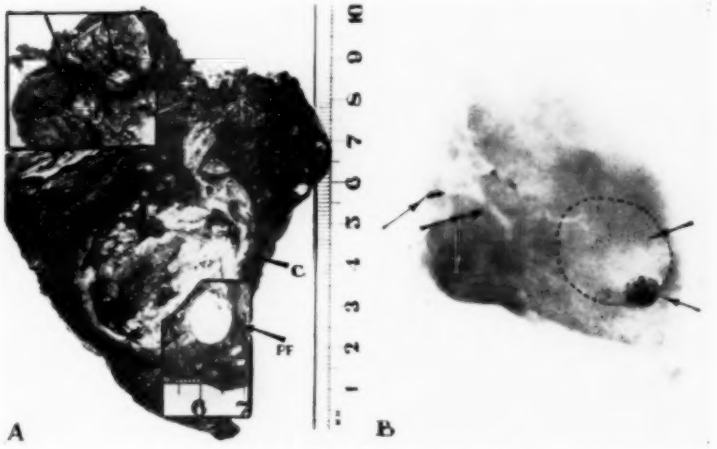

FIG. 6. Case 9. Female, 14 years old

A. Specimen: large, thick-walled cavity (C). Inset right lower: calcified focus (PF) found in dorsal aspect of cavity wall. Inset left upper: small, calcified lymph nodes.

B. Roentgenogram of specimen: outlines of cavity with calcified focus (corresponding to PF in figure 6A). To the left: calcification in hilar region.

Resected specimen: The right lower lobectomy specimen obtained in September 1949 contained a large cavity, 4 cm. in diameter, in the anterolateral aspect just above the base. The cavity was lined by a thick, caseous wall in its central part and filled with caseous material in its dorsal part. In the latter, a well-encapsulated, chalky, caseous pneumonic focus, 0.8 by 1 cm., was found to form part of the cavity wall (figure 6A, right inset). Two regional bronchopulmonary calcified lymph nodes (0.4 by 0.3 cm. each) were seen (figure 6A, left inset).

Comment: The picture was that of a lesion of the primary complex type. In this case, the pulmonary component showed up as a cavity. It was still solid caseous, however, in its dorsal parts, where a calcified focus was found. Its association with calcified areas in the regional lymph nodes, particularly impressive in the roentgenogram of the specimen (figure 6B), suggested this to be the actual and original primary focus. The cavitated area appeared to be due to a local

extension of the primary focus, which progressed to cavitation while its source, the primary focus, calcified.

Case 10

A symptomless woman, aged 27, on routine roentgenographic examination during pregnancy, was shown to have an opacity in her right lower lobe. This was examined roentgenographically from time to time. The opacity remained stationary, her general condition was excellent, and she had no symptoms of any sort. Excision of the lobe was undertaken as the patient was very apprehensive.

Resected specimen: The specimen showed a subpleural, old caseous circular focus, 2½ by 3 cm., with a small, central cavity (figure 7). Histologically, a caseous pneumonia was present, with preservation of the elastic alveolar pattern in places. A roentgenogram of the specimen revealed a considerable amount of patchy calcification in the focus (figure 7, upper inset). The histologic changes of liquefaction included edema and the influx of polymorphonuclear and mononuclear cells, as well as the presence of large numbers of tubercle bacilli. From the dissection of the specimen itself and from the clinical chest roentgenograms, no information could be obtained concerning the site of primary infection. Nevertheless, some hard, calcified lymph nodes found at operation in the hilar parts regional to the tuberculoma suggested that primary infection had taken place in this area.

Comment: This case is a further example of the occurrence of liquefaction in a calcifying focus. The close local association of these changes is regarded as an indication of recrudescence.

GENERAL COMMENT

A number of post-mortem and lobectomy specimens have been presented with "early phthisical" changes. These revealed the actual or potential source of bronchogenic spreads, namely, liquefying areas with numerous acid-fast bacilli or a fully developed cavity. Phthisis was not far advanced and an opinion could thus be formed concerning its relationship to primary infection. It appeared to be separated from primary infection by a long interval. This was recognizable in the history of those cases in which a typical concomitant of primary infection, such as pleural effusion, antedated phthisis by many years (for example, nine years in *Case 4*).

The anatomical diagnosis of recrudescence was based on the hard calcified changes occurring in the same focal area as soft and liquefying caseous material. The intimate mixture of such hard and soft areas resulted in a porridge-like consistence and was, indeed, the feature common to the cases described. Liquefaction occurring in close proximity to calcification indicates activity and progression of a partially healed focus, i.e., recrudescence.

A porridge-like ("pultaceous") consistence of a focus alone is also seen in cavities healed by inspissation of contents and without recrudescence. Usually, especially in lobectomy cases, roentgenograms will be available to show that the solid focus seen in the specimen is a cavity healed by inspissation of contents. In doubtful cases, histologic examination often helps. Preservation of elastic fibers in an alevolar pattern, as seen in the cases described in the present report, is not usually found in healed cavities. In the latter, the bronchus is obliterated; whereas, in recrudescent foci it need not be so.

It should be noted, however, that an inspissated cavity can form the source of phthisis in the same way as a recrudescent focus. The following case is an

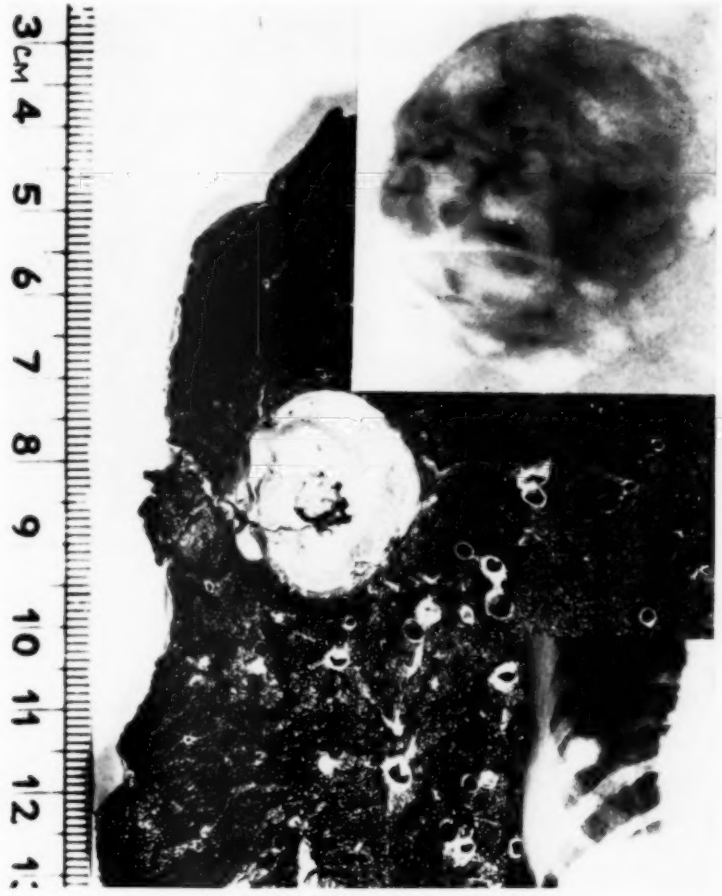

FIG. 7. Case 10. Female, 27 years old

Large, circular, old caseous focus with central cavity. Upper inset: roentgenogram of specimen; focus with patchy calcification. Lower inset: focus in a chest roentgenogram prior to lobectomy.

example in which phthisis was traced to the cavitating primary focus ("primary cavity") which had been converted into a healed "pultaceous" focus at the time of observation.

Case 11

In a male, aged 44, a cavity in the lingula region of the lung was seen in April 1946 and interpreted as "lung abscess" (figure 8A). This became smaller and converted into a solid focus within a month (figure 8B). Roentgenograms taken in April 1948 revealed extensive bilateral tuberculosis with systems of cavities, especially in the left upper lobe. A small, hard nodule was then recognizable in the area corresponding to the original cavity (figure 8C).

Post-Mortem Findings

At necropsy (No. 48/224), careful slicing and roentgenography failed to reveal any calcified changes other than the chalky primary complex in the left upper lobe (see below). An extensive cavitating fibrocaseous tuberculosis was found in both lungs with a thick-walled giant cavity in the left upper lobe (figure 9). In the lower anteromedial parts of the

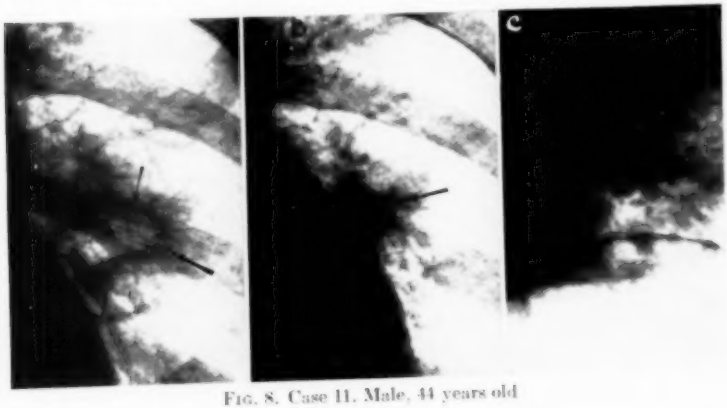

FIG. 8. Case 11. Male, 44 years old

A. April 9, 1946. Cavity in lingula region.
B. April 30, 1946. Cavity converted into a solid focus.
C. April 1948. Small, hard nodule in the area of the original cavity.

left upper lobe near the hilum, a well-encapsulated, chalky focus was found (2 by 1 cm., figure 9, arrow). A corresponding lower tracheobronchial lymph node contained a well-defined encapsulated, chalky focus, 0.3 cm. in diameter (figure 9, inset, arrow).

Comment: The chalky focus in the lower part of the left upper lobe corresponded in situation and size to the lesion in the lingula region seen in April 1946. At that time, it was a cavity in a state of shrinking and conversion into a solid focal shadow within one month. The anatomic change is consistent with a cavity "healed" by conversion into a solid focus. Its association with a similar focus in the corresponding lymph node and the absence of calcified changes in the lungs mark it as the primary focus or, more precisely, as a healed primary cavity. In spite of its local regression and healing, it must have laid the seeds for the progressive consumptive lesion with a fatal issue after two years. For in addition to the clinical follow-up, the lung changes, especially the thick, fibrocaseous cavity walls, suggested a protracted development and course.

In other words, here is an instance of phthisis developing by bronchogenic aspiration from a healed primary cavity acquired at the age of forty-four. Such phthisical evolution from a cavity which, by the time of observation, has healed by conversion into a solid focus has been described before by Pagel and Toussaint (3) with the difference, however, that the instance published previously was of a healed postprimary cavity.

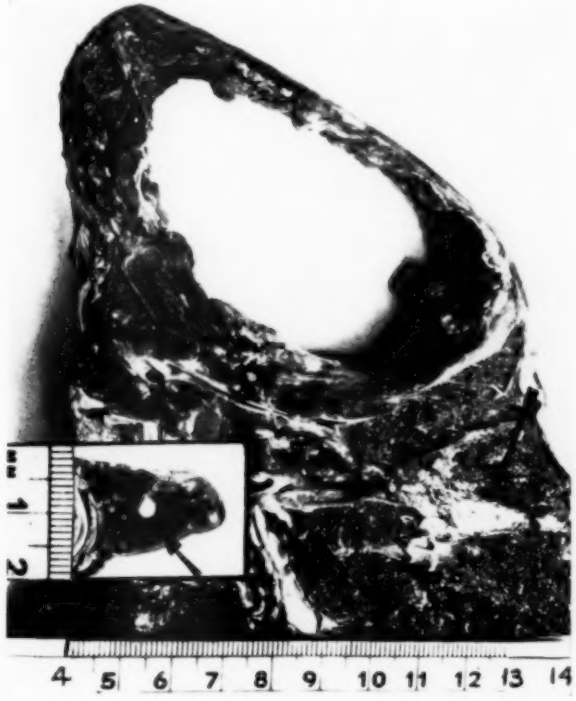

FIG. 9. Case 11

Specimen of left upper lobe with chalky focus (arrow) in the subpleural parts below a large cavity. The focus corresponds to the cavity seen originally in the lingula. Inset: lower tracheobronchial lymph node with a well-defined, small, chalky focus; the lymph node part corresponding to the healed primary cavity.

In a further observation, the changes are equivocal. They could be interpreted either as recrudescence or as its simulation by an inspissated cavity.

Case 12

A woman, aged 29, was well until April 1948, when she noticed that she was becoming extremely lethargic; to perform an ordinary day's work was difficult. She suffered from

anorexia and developed pain in the back on both sides. In August 1948, a chest roentgenogram revealed a rounded, clearly-defined opacity in the left lower lobe. The sputum smear showed no acid-fast bacilli.

Resected specimen: A lobectomy was performed. In the anterior apical part of the left lower lobe, a roughly spherical, caseous mass was found, approximately 3 cm. in diameter, which extended in places to within 0.5 cm. of the pleura (figure 10). Further sectioning showed a number of small, round foci between the main focus and the pleura, and at one

FIG. 10. Case 12. Female, 29 years old

Lobectomy specimen: large chalky focus near apex of left lower lobe. Lower inset: roentgenogram of specimen showing patchy calcification. Upper inset: chest roentgenogram prior to lobectomy; round focus in left lower mid-zone.

point there appeared to be a caseous bronchus adjacent to the main focus. A roentgenogram of the specimen revealed the extent of patchy calcification in the focus (figure 10, lower inset).

Histologic examination showed that the large focus consisted of caseous material surrounded by a thick, fibrous capsule. Centrally, it showed areas of liquefaction. Near one of the liquefied areas was a fragment of bronchial cartilage. The adjacent focus, thought to be a bronchus, was separated from the large focus by a few bronchioles and vessels. It showed a thick, fibrous capsule and a caseous center with no active cellular reaction.

Elastic staining showed remnants of elastic structures of vessels but no alveolar septa in the large focus. A few small, satellite tubercles were outside the large focus and a few perivascular fibrous scars were present in the surrounding normal lung tissue. Large numbers of acid-fast bacilli were seen in the liquefying areas of the main focus.

Comment: The case is one of lobectomy, in which the lobe contained a tuberculoma believed to be an inspissated cavity. No roentgenograms were available to confirm this impression but the absence of elastic fibers in an alveolar pattern supported it. On the other hand, the close proximity of calcified debris and areas of acellular liquefaction with acid-fast bacilli suggested recrudescence. At all events, the mere presence of considerable numbers of acid-fast bacilli showed that this was not firmly healed but was still an active source of bronchogenic propagation of the tubercle bacillus. It may, therefore, be designated as an inspissated cavity with signs of recrudescence.

Another possible source of confusion is the accidental involvement of preexisting calcified nodules by a recent and independent lesion. As a rule, histologic examination will clear up this point satisfactorily. In the present cases, invariably one and the same focal area was found to be enclosed by a thick, fibrous capsule and to contain calcified, as well as caseous and liquefying, areas. Presence of liquefaction, i.e., the very opposite of focal healing, is the point most important in the diagnosis of recrudescence. This is particularly striking when all stages of liquefaction are seen in a partly calcified focus from the "acellular" simple edema and disintegration around collections of acid-fast bacilli to the massive infiltration and dissolution of caseous and calcified material by swarms of polymorphonuclear and mononuclear cells. On the other hand, partial calcification does not necessarily suggest recrudescence. It only indicates an incomplete and drawn-out process of healing. Only when roentgenographic follow-up observations suggest growth of the solid focus by peripheral extension of caseous matter, in spite of patchy calcification in the more central parts, can recrudescence be suspected.

In the present material, recrudescence had occurred in primary, as well as in postprimary, foci. The latter were not due to reinfection since they were not associated with changes in the regional lymph nodes. They were, however, very much like the primary focus in firmness and quality (calcification), and it is reasonable to suppose that they dated from an early postprimary phase. Recrudescence in these foci, therefore, bridged the long gap between primary infection and phthisis. In some of the cases, recrudescence could be attributed to intervening nonspecific factors, such as diabetes, carcinomatosis, and starvation. Such incidents are known to weaken resistance against tuberculosis. It is tempting to assume that these factors were responsible for an initial multiplication of tubercle bacilli. For, in the early stages, the only change seen was a collection of tubercle bacilli in those areas where the calcified ground substance appeared to be disintegrated by an influx of fluid. The bacilli at this stage were mostly granular and stained pink with Giemsa, i.e., were not fully acid fast. When liquefaction had fully developed, i.e., where inflammatory cells were conspicuous, fully acid-fast tubercle bacilli were seen, which stained faintly blue with Giemsa.

Summary

Twelve observations of early bronchogenic phthisis, due to recrudescence of old foci, have been described, 6 in post-mortem, and 6 in lobectomy, specimens.

The picture common to these observations was that of "pultaceous liquefaction." This was due to an intimate mixture of retrogressive (calcifying) and progressive (liquefying) changes. It indicated reanimation of old foci whereby these became an actual or potential source of bronchogenic aspiration of tubercle bacilli.

Recrudescence must be distinguished from (a) cavity healing by inspissation of contents and (b) accidental inclusion of calcified changes in a new and independent process.

Conversion of quiescent into active and progressive foci occurred in primary, as well as in postprimary, lesions.

Intervening disease, such as diabetes, starvation, and carcinomatosis, appeared to be responsible for the flaring-up and phthisical development of old foci.

Sumario

La Recrudescencia en la Tisis Incipiente. Estudio de Ejemplares Autópsicos y Lobectómicos

Describense 12 observaciones de tisis broncógena incipiente debida a recrudescencia de focos antiguos: 6 en ejemplares autópsicos y 6 en lobectomizados.

El cuadro compartido por todas estas observaciones fue de "licuación sebácea", debiéndose a una mezcla íntima de alteraciones regresivas (calcificantes) y progresivas (licuefacientes). Esto indica reavivación de focos antiguos, convirtiéndose así en causa real o potencial de aspiración broncógena de bacilos tuberculosos.

Hay que diferenciar la recrudescencia de: (a) cierre de las cavernas por espesamiento del contenido y (b) inclusión fortuita de alteraciones calcificadas en un proceso nuevo e independiente.

La transformación de focos quiescentes en activos y evolutivos ocurrió en lesiones tanto primarias como postprimarias.

La intervención de alguna enfermedad, tal como diabetes, inanición o carcinomatosis, pareció ser la causa de la exacerbación y del desarrollo tísico de los focos antiguos.

Resume

La réactivation dans la tuberculose pulmonaire au début. Une étude de pièces d'autopsie et de lobectomie

Douze observations de tuberculose broncho-pulmonaire au début, déterminée par le réveil d'anciens foyers, ont été décrites, d'après 6 pièces d'autopsies et 6 de lobectomies.

Dans ces observations l'aspect habituel était une "liquéfaction sébacée". Ce caractère résulte de l'association de modifications régressives (calcification) et progressives (liquéfaction). Cet aspect indique le réveil de vieux foyers, qui deviennent ainsi une source effective ou potentielle d'aspiration bronchique de bacilles tuberculeux.

Cette réactivation doit être distinguée: (a) de la cicatrisation d'une cavité par assèchement du contenu et (b) de l'inclusion accidentelle de calcifications au cours d'un processus nouveau et indépendant.

La conversion de foyers, "au repos", en foyers actifs et évolutifs s'est produite dans les lésions primaires comme dans les lésions ultérieures.

Les affections intercurrentes, telles que le diabète, la malnutrition, et le cancer, paraissent être responsables des poussées et du développement de la tuberculose pulmonaire à partir de vieux foyers.

Acknowledgment

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OBSERVATIONS ON THE EFFECT OF AMITHIOZONE (TIBIONE) IN SELECTED TUBERCULOUS PULMONARY LESIONS[1]

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INTRODUCTION

The German investigators, Robert Behnisch, Fritz Mietzsch, and Hans Schmidt (1) reported chemical studies on thiosemicarbazones with particular reference to antituberculous activity, and Gerhard Domagk (2) reported investigations on the antituberculous activity of the thiosemicarbazones *in vitro* and *in vivo*. Anton Mertens and Rolf Bunge (3) reported on the beneficial effect of amithiozone[2] on various types of tuberculosis in man. They first observed that recently developed exudative pulmonary lesions showed unusually rapid recession with concomitant improvement in symptoms and general condition. They also observed that cavernous lesions responded impressively. Hinshaw and McDermott (4), in reviewing the German experience, observed a large number of cases in which an impressive degree of roentgenographic clearing of pulmonary lesions had occurred during relatively prolonged amithiozone therapy—four, six, and nine months. The authors were unwilling, however, to relate the results to the drug therapy with absolute certainty. Results of chemical, *in vitro*, and *in vivo* observations have subsequently been confirmed by a number of investigators, and since the German reports appeared there have been a number of observations on the effect of thiosemicarbazones in man and on the toxic effects resulting from its administration. Yet, few of these report the effect on pulmonary lesions per se. Martini, Moers, and Gansen (5) were able to evaluate the therapeutic effects of amithiozone on 94 patients in a series of 240. The disease was influenced favorably by the drug in 72 of the 94 patients observed. These writers contend that there was definite proof of therapeutic effect in exudative, productive, and mixed types of disease. In 56 of 87 patients with cavities refractory to general treatment, they observed regression, even of large cavities. Livingstone and Street (6), in a small series, observed improvement in exudative pulmonary lesions; but little, if any, benefit was observed in the more chronic type of lesions. Sandhaus, Jenkins, Burdon, and Halpert (7) studied the effects of amithiozone treatment of pulmonary tuberculosis on 21 patients with various types of lesions, moderately or far advanced, for one hundred and twenty days. Ten were treated with amithiozone alone. Five improved and five became worse. In 11 patients treated with amithiozone and streptomycin, all improved. Belgorod, Alexander, Meidt, and McGaley (8) state

[1] From the Division of Tuberculosis, Grasslands Hospital, Valhalla, New York.

[2] Amithiozone (Tibione) used in these investigations was contributed by Schenley Laboratories through the courtesy of Dr. Charles Dutchess, Medical Director. Tibione is the proprietary name of Schenley Laboratories for amithiozone.

in a recent report that amithiozone seems to be less effective when used alone than when used in combination with streptomycin and para-aminosalicylic acid.

Observations on the effect of amithiozone in guinea pigs: Two separate animal experiments, as a part of the present study, were reported in August 1950 (9) and in March 1951 (10). The first showed that, in the limited type of experiment performed and with the dosage employed, amithiozone proved to be as effective an agent against tuberculosis in guinea pigs as streptomycin. Combined therapy with amithiozone and streptomycin in one-half of the dose employed, as was the case when either drug was used alone, was equally effective as amithiozone alone and possibly more effective than streptomycin alone. In the second report, under the conditions of the experiment, amithiozone in combination with a subeffective dose of dihydrostreptomycin proved to be more effective as an antituberculous agent in guinea pigs than the combination of para-aminosalicylic acid (PAS) with a subeffective dose of dihydrostreptomycin. The former combination was also more effective than amithiozone used alone.

PLAN OF INVESTIGATION

Because of reports on toxicity of the thiosemicarbazones in man, in the present study amithiozone was first used in patients with far advanced progressive disease, some with complications such as endobronchial disease and laryngitis, who were discharging tubercle bacilli resistant to streptomycin. Admittedly, advanced disease of this type is not the most desirable type for clinical evaluation of any therapeutic agent. This view is supported by the fact that there was progression of the disease in spite of adequate supportive treatment combined with the use of streptomycin and para-aminosalicylic acid. As will be described later, some toxic symptoms were observed; yet some patients showed mild clinical improvement with reduction of fever. An early observation was the improvement in mucous membrane lesions that no longer were benefited by streptomycin and para-aminosalicylic acid. After an orientation period, when toxicity was under study and when it was considered safe to administer amithiozone in daily dosage of reduced amounts (100 to 150 mg.), it was used in selected cases of pulmonary tuberculosis. Clinical evaluation was made independently by three of us (W. G. C., J. W. N., and R. H. O.), and the laboratory studies were directed by D. M. S. Although there were differences of opinion regarding the degree of roentgenographic clearing and clinical improvement from time to time, all were in general agreement regarding its ultimate effect.

Case material: A total of 45 patients were treated for various periods of time from January 1950 through December 1951. The ages of the patients varied from 17 to 76 years: 3 were 20 years or less; 12, from 20 to 30; 19, from 31 to 40; 4, from 41 to 50; 4, from 51 to 60; and 3 were more than 60 years of age. Twenty-five were females (15 white, 10 Negro) and 20 were males (12 white, 8 Negro). Twenty-nine of the 45 had previously been treated by streptomycin. In 15 patients, the organisms in the sputum showed some degree of streptomycin resistance, and in 11 the lesions had ultimately failed to respond to streptomycin therapy. Sixteen of the group had previously had no chemotherapy. Of the 45 patients, 12 had acute or chronic disease (4 moderately advanced and 8 far advanced) with no previous treatment other than bed rest. Seven patients (3 with moderately advanced and 4 with far advanced disease) had had recent acute spread of disease. In none of these were the tubercle bacilli resistant to streptomycin, although in some the drug had previously been administered. Twenty-three patients (4 moderately advanced and 19 far advanced) had progressive downhill courses not amenable to definitive treatment other than chemotherapy. Fourteen of these (2 with moderately advanced and 12 with far advanced disease) had organisms resistant to streptomycin. Two patients had stable pulmonary disease, but active endobronchial disease; in one the tubercle bacilli were resistant to streptomycin. One patient had chronic lymphohematogenous tuberculosis.

Regimen: At the start of the study, all of the patients received 200 mg. of amithiozone per day in two doses. The dose was reached by gradually increasing the dosage over a period of two weeks. In May of 1950 the dosage was decreased to 150 mg. per day for patients over 110 pounds, and 100 mg. per day for those under 110 pounds. The change was made on the basis of toxicity observations and reports from other sources. Length of treatment was randomized by using the numbers on the roentgenograms. The course of treatment was six months for those with odd numbers and one year for those with even numbers. At the time of the present report,[3] 5 patients had been treated for one month or less; 3 had received three months' treatment; 22 had received from five to six months' treatment; 3 had received nine months' treatment; and 12 had been treated for one year (or approximately one year).

Treatment was discontinued permanently in 8 patients because of drug toxicity; in 2 of these patients, treatment was stopped after eleven and one-half months and was not started again because it was not considered necessary. Two patients refused further treatment after three months. Of the 8 patients who received less than six months of treatment, treatment of 4 was discontinued because of drug toxicity, one because of increase following hemoptysis; 2 because of refusal to accept further therapy; and one because of extrapulmonary disease which had not been controlled although the pulmonary disease had improved during amithiozone treatment.

RESULTS

Therapeutic Studies

On the basis of clinical and roentgenographic changes, the following evaluation was made. Of the 45 patients, 27 showed some improvement during treatment; 8 showed no significant change; and 10 became worse. In general, it can be said that the improvement was gradual and somewhat delayed in onset in comparison with the rapid improvement seen in streptomycin or streptomycin and para-aminosalicylic acid therapy. The group may be divided roughly into those treated six months or less (30 patients); and those treated for approximately a year (15 patients). In both groups 60 per cent showed improvement, so no over-all conclusion can be drawn as to the value of the two regimens.

Pulmonary disease: Forty-two patients were treated primarily for active pulmonary disease. Of 15 of these treated for approximately one year, 9 showed improvement (2 had moderately and 7 far advanced disease). Case 13 (figure 1) and Case 23 (figure 2) are examples of acute disease which increased under observation and then improved during a year's course of amithiozone. Case 17 (figure 3) represents a more chronic type of disease which responded more slowly during twelve months of treatment. Twenty-seven patients received approximately six months of amithiozone for pulmonary disease (9 with moderately and 18 with far advanced disease). Of these, 17 showed some improvement. Case 20 (figure 5) is an example of improvement following a six months' course of

[3] December 31, 1951.

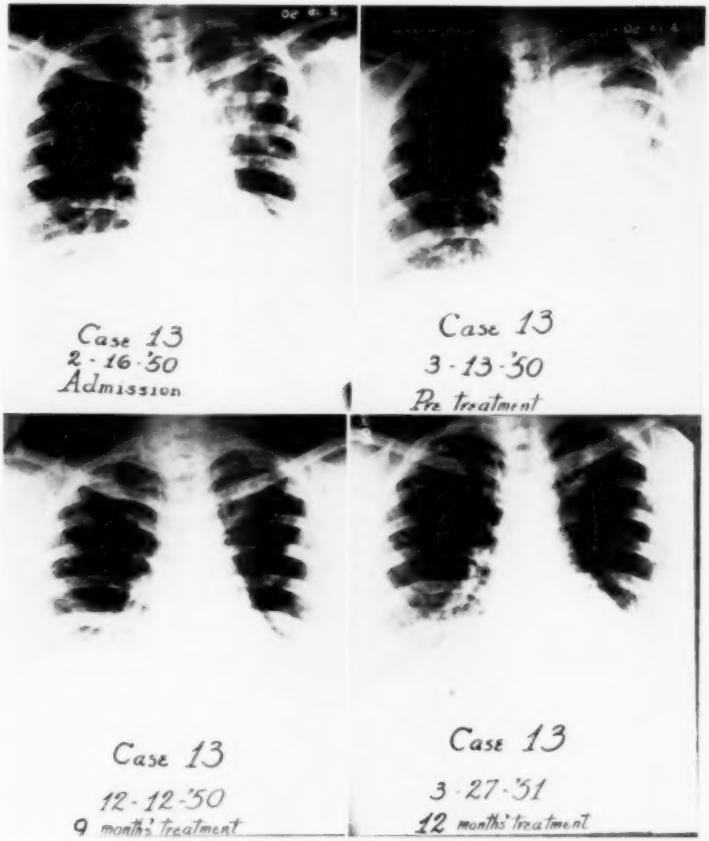

FIG. 1. Case 13 (L. B.)

Roentgenogram dated February 16, 1950, showing a 34-year-old Negro woman, readmitted in February 1950 with recent disease in the left lung. She had previously been treated in 1948 for a minimal lesion on the right.

Roentgenogram dated March 13, 1950, showing a marked increase throughout the left lung following negative bronchoscopy and one month of bed rest. Amithiozone was started and continued for 365 days, at 150 mg. per day. There was progressive roentgenographic clearing, and sputum became negative for tubercle bacilli on concentrate in the third month; on culture, in the fourth month.

Roentgenogram dated December 12, 1950, showing the marked clearing after nine months.

Roentgenogram dated March 27, 1951, taken at the end of treatment, showing a cavity which undoubtedly had been present before. The sputum culture was again positive for *M. tuberculosis* at this time, and the organisms were sensitive to amithiozone *in vitro*. Within two weeks of discontinuance of treatment, the patient suddenly developed laryngitis and pericavitary spread of disease.

FIG. 2. Case 23 (M. W.)

Roentgenogram dated May 15, 1950, taken on admission, of a 27-year-old Negro female who had had a normal roentgenogram one year previously.

Roentgenogram dated June 9, 1950, showing spread of disease following a mild hemoptysis after three weeks on bed rest. Amithiozone was started at this point, with progressive improvement and "conversion" of sputum concentrate.

Roentgenogram dated April 9, 1951, taken just before left pneumothorax was instituted after ten months of treatment when only alternate cultures were positive for *M. tuberculosis* and the organisms were sensitive to amithiozone, which was discontinued after one year. Patient also had slight inflammation of the left upper lobe bronchus on admission, which cleared completely on amithiozone.

the drug. A number of cases showed some initial clinical, symptomatic, and even roentgenographic improvement, but later the disease relapsed with the emergence of drug-resistant organisms in the sputum. Case 14 (figure 4) is an example of this group, with an excellent early response of an extremely acute illness to

FIG. 3. Case 17 (A. A.)

Roentgenogram dated June 20, 1949, taken on admission, of a 63-year-old white man who had known tuberculosis for four months. Disease increased in August 1949 following operation for strangulated inguinal hernia.

Roentgenogram dated April 20, 1950, taken at the beginning of amithiozone treatment.

Roentgenogram dated April 30, 1951, taken at the end of one year's treatment during which patient showed progressive improvement and was allowed to become ambulatory. Sputum concentrate was negative for tubercle bacilli after one month's treatment. Cultures were occasionally positive after three months of treatment. By December 1951 the patient had been discharged with continuously "negative" cultures and inactive disease. No significant toxic effects from chemotherapy.

amithiozone, with subsequent relapse and spread of disease with the appearance of drug-resistant organisms in the sputum.

Endobronchial disease: Two patients were treated primarily for endobronchial disease. One of these (Case 12) was treated for six months with excellent results. The other patient, treated for endobronchial disease alone, had received only one month of therapy when severe gastrointestinal distress and increased bromsulfalein retention necessitated discontinuance of amithiozone permanently. Nine

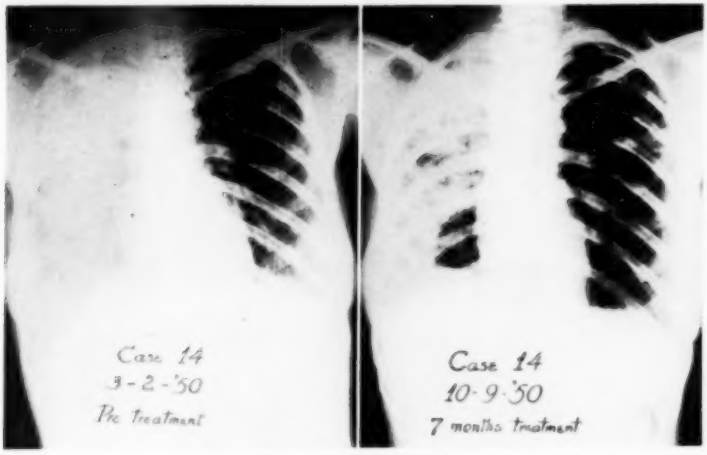

FIG. 4. Case 14 (E. L.)

Roentgenogram dated March 2, 1950, admission film of a 20-year-old Negro female who had had normal chest films in November 1949 and whose amithiozone treatment was started on admission. Her course was quite toxic with temperature to 103°F. Despite nausea, vomiting, and temporary liver damage (enlarged liver, 26 per cent bromsulfalein retention, and 4 plus cephalin flocculation test), which cleared completely with discontinuance of drug and resumption at a lower dosage, patient had marked progressive clinical improvement with normal temperature until the end of six months' treatment.

Roentgenogram dated October 9, 1950 shows the condition at the end of treatment when the disease increased on the left and fever was present. This occurred after the organisms in the sputum were persistently resistant to amithiozone after four months of treatment.

patients with active pulmonary disease also had active tracheobronchitis at the time of amithiozone treatment. In 7 of these, improvement in the bronchial lesion occurred during treatment. In 2 of these 7 cases, the improvement of endobronchial disease continued despite later worsening of the pulmonary lesions. In 2 cases the bronchial disease showed no change.

Chronic hematogenous disease: The one case of extrapulmonary chronic hematogenous disease was treated primarily to observe the effect on a draining tuberculous cervical lymph node. No change was noted in the draining sinus. This man had a severe anemia before treatment was started and, as his erythrocyte count showed a further reduction during treatment, it was considered

unsafe to continue amithiozone and the treatment was discontinued after one month. He died one month after cessation of treatment.

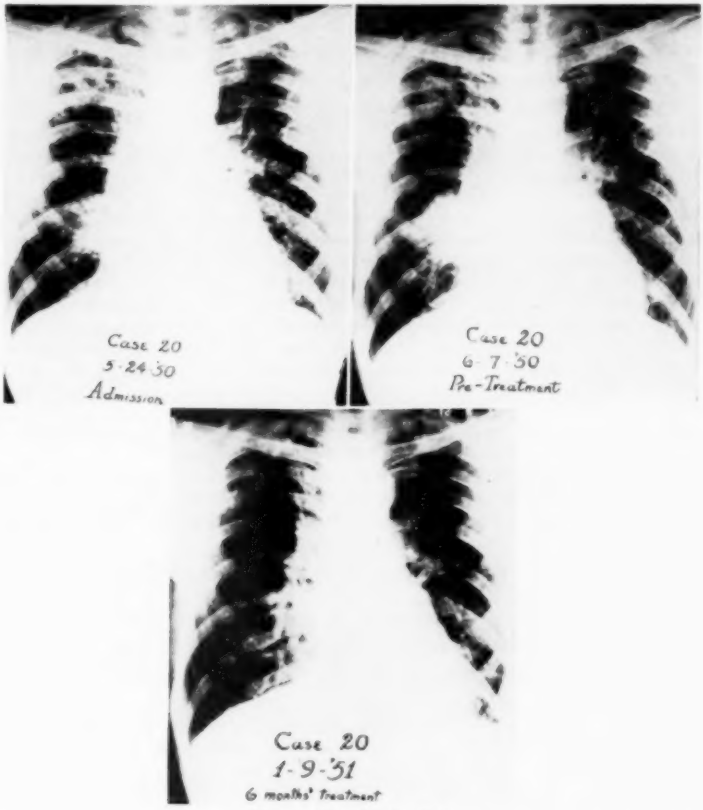

FIG. 5. Case 20 (L. C.)

Roentgenogram dated May 24, 1950, admission film of a 50-year-old white male with four months' symptomatic history.

Roentgenogram dated June 7, 1950, showing an increase at the right base which occurred while patient was on bed rest. Amithiozone was started at this time and was continued for more than six months, with symptomatic improvement.

Roentgenogram dated January 9, 1951, shows condition at the end of treatment. Sputum still positive for tubercle bacilli on smear and the organisms were sensitive to amithiozone.

Ultimate outcome: Of the 45 patients in the group, 7 have since died; 8 continued with active disease in approximately the same condition; 4 are much worse than at the end of treatment; 4 are still under treatment (one continued over one year); and 9 have what may be considered to be arrested disease (4 of these

on amithiozone and bed rest alone; 5 with subsequent collapse, surgery, and/or streptomycin and para-aminosalicylic acid). Eleven patients, still in the hospital, are better but their lesions are not yet arrested. Two patients have received a second six months' course of amithiozone because of relapse. Both responded somewhat to the second course, but continue to have active disease with sputum positive for *M. tuberculosis* on culture.

Case Report

Case 12 (A. D). This 35-year-old white woman was admitted in October 1949, with wheeze and sputum recently positive for *M. tuberculosis*. Bilateral fibrocalcific inactive-appearing infiltrations, discovered in 1948, showed no change. No roentgenograms are reproduced because there was no change whatever throughout her course of treatment. The sputum remained positive for *M. tuberculosis* on bed rest, and four months later bronchoscopy showed "fairly extensive and deep lesion on the anterior tracheal wall, extending from 3 cm. below the glottis approximately to the beginning of the right main bronchus." Repeat bronchoscopy two weeks later revealed "ulcerative lesions in the trachea on the right above the carina; also a granulating type of lesion just within the opening of the right upper lobe bronchus." At this point amithiozone was started and continued for a total period of six months. Within seven weeks a loud groaning rhonchus previously heard all over the chest had disappeared, and bronchoscopy showed "lesion almost entirely gone. Some thickening present but no ulceration." Sputum cultures became negative for tubercle bacilli, and bronchoscopy at the end of treatment showed "no evidence of endobronchial disease." Improvement continued until September 1951, when a sputum culture was again positive for tubercle bacilli and re-bronchoscopy showed "heavy white exudate clinging to the bronchial wall of the right main stem bronchus. Narrowing of the right upper lobe bronchus without ulceration and granulation, but induration of the mucosa." No tracheal lesion was seen.

Drug Toxicity

Gastrointestinal: Toxic manifestations of various degrees of seriousness caused by amithiozone have frequently been observed by all investigators, and hepatotoxicity is cause for great concern and constitutes the greatest threat to amithiozone's usefulness.

Symptomatically, fewer unpleasant effects of toxicity were observed with amithiozone than with para-aminosalicylic acid. Nausea or vomiting occurred in 17 patients. In 14 of these the distress present was slight and did not warrant discontinuing the drug. In some of the patients it occurred only on one or two occasions. Ten patients developed nausea and vomiting while on the earlier higher dose of 200 mg. per day, but had no further distress when the dose was decreased. Three patients had severe nausea and vomiting. In one they occurred concomitantly with liver damage and may have been due to this rather than to the gastric irritation per se. In another some liver damage also occurred, but the nausea and vomiting preceded it, and was severe enough to necessitate stopping the drug entirely. In still another case (Case 14) the nausea, vomiting, and liver damage caused temporary discontinuance of the drug while the high dose was being given. Following a few weeks without medication, this patient continued at lower dosage with no recurrence of toxic symptoms.

Although nausea and vomiting were slight, a decrease in appetite was noted

in some patients. In no instance was the immediate marked increase in appetite, so frequently seen with streptomycin treatment, observed in patients receiving amithiozone. Only 18 of the patients gained weight and the average gain was 7.7 pounds. Twenty-three patients lost weight and, in some, this occurred despite roentgenographic and clinical improvement in disease.

Allergic reactions: Rash and itching of the skin caused some discomfort, but in most cases it was fleeting and responded to antihistaminics. Nine patients developed a rash; in 6 it was accompanied by itching. One patient had itching without a demonstrable rash. In only 2 patients were rashes severe enough to necessitate cessation of treatment. In one of these the rash was accompanied by fever, apparently due to amithiozone.

Hepatic dysfunction: Liver dysfunction seems the most serious of the toxic reactions to amithiozone. The patients were followed with monthly determinations of:

1) Percentage of bromsulfalein (BSP) retention forty-five minutes after intravenous injection of 5 mg. per kg.
2) Albumin and globulin determination on serum
3) Cephalin flocculation tests
4) Icterus index determination

Other procedures were performed as necessary.

The most frequent evidence of liver dysfunction was an increase in bromsulfalein retention. Twenty-six patients in all had an increase in bromsulfalein retention of more than 5 per cent above pretreatment levels. Smaller increases were considered as clinically insignificant or within the range of laboratory error. Of these 26 patients, 12 showed an increase of more than 10 per cent. In 14 of the 26 cases, the degree of bromsulfalein retention returned to normal despite the continued use of amithiozone. In 10 cases it returned to pretreatment levels after the drug was discontinued. In one of these, the drug was discontinued temporarily and then re-started with lower doses. In others, the bromsulfalein retention occurred at the end of the treatment period. In the 2 patients who died after amithiozone was discontinued, the bromsulfalein test had not been repeated before death. Table 1 gives this information in tabular form.

One young white female developed sporadic glycosuria and hyperglycemia at the end of five months' treatment with amithiozone. Although there was a history of diabetes in the family of this patient, the glucose tolerance test was not typical of diabetes mellitus. The blood sugar dropped to low levels soon after the initial excessive rise following the ingestion of 100 gm. of glucose. When amithiozone was discontinued, the glycosuria and hyperglycemia disappeared completely and the glucose tolerance test returned to normal. There was increased bromsulfalein retention at the time of hyperglycemia, and it was assumed that this abnormality of sugar metabolism was due to liver dysfunction secondary to amithiozone toxicity. This case was the only one in which such findings occurred.

Cephalin flocculation tests became positive or more strongly positive in 7 patients during the course of treatment. In 3 of these patients, the cephalin

flocculation tests had previously been positive, although they were negative at the time treatment was started. In 2 of these, there was no concomitant increase in bromsulfalein retention. Only one patient had a significant increase in icterus index, up to 11 units. This patient was one who had evidence of severe liver damage, for which reason amithiozone was discontinued permanently.

No significant changes were noted in total protein, albumin, or globulin concentrations. This is in contrast to the findings of Belgorod and co-workers (8), who found a persistent increase in total protein which seemed attributable to an increase in globulin. In the present study there were more patients whose globulin decreased than those whose globulin increased under treatment. The majority of patients showed a slight increase in serum albumin. This might be

TABLE 1

THE INCIDENCE AND DEGREE OF INCREASE IN BROMSULFALEIN RETENTION DURING TREATMENT WITH AMITHIOZONE OVER PRE-TREATMENT LEVELS*

TOTAL INCREASED OVER 5 PER CENT†		OF THESE—						RETURN TO PRETREATMENT LEVELS—					
		Increased 6 to 10 per cent		Increased 11 to 20 per cent		Increased over 21 per cent		During treatment		Treatment discontinued		Did not return	
Number	Per cent‡	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
26	58	14	31	7	16	5	11	14	31	10	23	2	4

* Bromsulfalein retention tests drawn routinely pretreatment and monthly during treatment, more frequently if retention is increased.

† This represents per cent retention above pretreatment level and not absolute retention, as pretreatment retentions varied from 0 to 12 per cent.

‡ Of 45 patients.

Figures represent percentage of bromsulfalein retention in peripheral blood forty-five minutes after the intravenous injection of 5 mg. per kg. of body weight.

attributed to improvement in the general condition rather than to amithiozone therapy.

Hematologic studies: Complete blood counts and erythrocyte sedimentation rates were originally done once a week on all patients during the course of treatment. During the first month of treatment these studies were carried out twice a week; and, in any case in which there was significant change, more frequent blood counts were done. Later when it was found that the blood count did not reach dangerously low levels, the counts were taken once a week for the first month and thereafter every two weeks.

A consistent and almost universal mild anemia was found during the course of treatment. The erythrocyte count and hemoglobin concentrations dropped slowly during the first few months of treatment and then, for the most part, leveled off or began to increase again. Thirty-nine of the 45 patients showed a decrease of more than 250,000 cells per cu. mm. in the erythrocyte count over the pretreatment level at some time during therapy. In 34 of these, the counts subsequently increased again. When the pretreatment count is considered in

relation to the count at the end of treatment or at the time of writing the present report (December 31, 1951), 22 patients show a decrease in the erythrocyte count. These data are presented in table 2.

There was a similar change in the concentration of hemoglobin. Thirty-nine of the patients showed a drop in hemoglobin of 1 gm. or more per 100 ml. during treatment over the pretreatment level. In 24, the hemoglobin was below the pretreatment level at the end of treatment. This information is presented in table 3.

TABLE 2

DEGREE OF DECREASE IN ERYTHROCYTE COUNT UNDER AMITHIOZONE THERAPY

AMOUNT DECREASE	LOWEST DROP OVER PRETREATMENT LEVEL		SUBSEQUENT INCREASE IN ERYTHROCYTE COUNT WHILE UNDER TREATMENT		NUMBER WITH ERYTHROCYTE COUNT LOWER THAN PRETREATMENT LEVEL AT END OF TREATMENT	
	Number of patients	Per cent of 45	Number of patients	Per cent of 45	Number of patients	Per cent of 45
250,000–500,000/cu. mm.	5	11.1	4	8.9	7	15.6
500–1,000,000/cu. mm.	19	42.3	17	37.8	10	22.2
1,000–1,500,000/cu. mm.	11	24.4	9	20.0	4	8.9
1,500–2,000,000/cu. mm.	2	4.4	2	4.4	0	0
2,000–2,500,000/cu. mm.	1	2.2	1	2.2	0	0
>2,500,000/cu. mm.	1	2.2	1	2.2	1	2.2
Totals—	39 or 86.7% of 45 patients had drop in erythrocyte count during treatment.		34 or 75.6% of 45 patients had subsequent increase in erythrocyte count while under treatment.		22 or 48.9% of 45 patients show erythrocyte count below pretreatment levels at end of treatment (or at present time).	

While there were patients who had a drop in the leukocyte count during treatment, none developed a leukopenia of any significance. In only 9 patients did the blood count drop on any occasion below 5,000 and in only one was there one count below 4,000 cells per cu. mm. The leukocyte counts showed so much variation from week to week that no other significant changes were noted. There was also no consistent change in the polymorphonuclear leukocyte percentage. The lowest polymorphonuclear leukocyte differential count was 40 per cent except in one instance where a 29 per cent differential count was reported once.

In analysis of all cases, 23 patients had an increase in percentage of polymorphonuclear leukocytes and 22 had a drop in polymorphonuclear leukocytes

from beginning to end. In those patients who had a superimposed secondary infection during the course of amithiozone therapy, the leukocyte count and the polymorphonuclear leukocyte percentage increased, as would be expected.

Eosinophilia was not marked in any case. The highest eosinophile percentage encountered was 11 per cent, and only 13 patients had an eosinophile percentage of 6 per cent or more during treatment.

Erythrocyte sedimentation rate: There was no consistent effect on the erythrocyte sedimentation rate, although there were some surprising drops of the sedimentation rates which were not accompanied by other evidence of improvement.

Renal function: Urinalyses were done every two weeks during treatment and showed no significant change. Blood nonprotein nitrogen concentrations were determined once a month throughout the course of treatment, and no evidence of nitrogen retention was found.

TABLE 3

DECREASE IN HEMOGLOBIN LEVELS UNDER AMITHIOZONE THERAPY

AMOUNT DECREASE	LOWEST DROP OVER PRETREATMENT LEVEL		NUMBER WITH HEMOGLOBIN BELOW PRETREATMENT LEVEL AT END OF TREATMENT	
	Number of patients	Per cent of 45	Number of patients	Per cent of 45
1–2 gm./cu. mm.	6	13.3	14	31.1
2–3 gm./cu. mm.	17	37.8	5	11.1
3–4 gm./cu. mm.	12	26.7	3	6.7
4–5 gm./cu. mm.	2	4.4	0	0
>5 gm./cu. mm.	2	4.4	2	4.4
Totals—	39 or 86.7% of 45 patients had a drop in hemoglobin level during treatment.		24 or 53.3% show hemoglobin level below pretreatment level at end of treatment.	

Appearance of Amithiozone-Resistant Tubercle Bacilli

All patients had sputum or gastric specimens cultured for bacilli drug-sensitivity tests before treatment, at two-week intervals during treatment, and at the end of treatment. In some cases, further studies were done at varying intervals after treatment.

The specimens were prepared in the usual manner, using the sodium hydroxide method and neutralizing with phosphate buffer. During the first months of the study the specimens were first inoculated on Löwenstein egg-yolk medium. When growth was obtained in this, it was subcultured on Dubos liquid medium; then sensitivity tests were carried out, using one control tube of the Dubos liquid medium. The test cultures contained respectively: 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, and 125.0 γ of amithiozone per cc. of culture medium. The amithiozone was prepared in a 30 per cent solution of triethyleneglycol which was then introduced in proper dilution in the medium. The final concentration of triethyleneglycol in the medium did not exceed 1 per cent. Controls with this concentration of triethyleneglycol did not exhibit any antibacterial properties. Later in the study a method of studying resistance, which had given good results with streptomycin, was used. In this method, the concentrated specimen was inoculated directly on Löwenstein egg-yolk medium, using two control tubes containing no amithiozone and three test slants containing respectively

2, 10, and 50 γ of amithiozone per cc. of culture medium. For a while both methods were used for every specimen. The results with both methods were the same in so far as degree

TABLE 4

45 CASES STUDIED

In 8 patients sputum "converted" to negative cultures; in one of these, drug-resistant organisms appeared which later became sensitive before final disappearance.
In 15 patients resistant organisms appeared.
In 22 patients sputum remained "positive" on culture and tubercle bacilli were sensitive at end of treatment, or at present time.

Drug Resistance

CASE NUMBER	TIME OF APPEARANCE OF RESISTANT ORGANISMS FROM BEGINNING OF TREATMENT (SHOWN IN MONTHS)	DEGREE OF RESISTANCE TO AMITHIOZONE IN γ PER CC. OF MEDIUM*		REMARKS
		Liquid medium	Solid medium	
		greater than:	*greater than:*	
1	2	125.0	50.0	Still resistant one year after end of treatment.
2	4	125.0	50.0	Still resistant 4 months after treatment.
4	3	125.0	50.0	Resistant through 7 months' treatment.
14	4	31.25	2.0	Varied over 2 months with one sensitive culture.
16	10		2.0	1 specimen greater than 7.81 at third month; later resistant to greater than 10.
21	3½	125.0	2.0	At 7 months greater than 31.25; at 8 months less than 1.95; and "negative" thereafter.
25	4	125.0	10.0	Greater than 50 one month after treatment.
26	3½	62.25	50.0	Greater than 50 four months after treatment.
28	6	15.62	2.0	At end of treatment.
31	2½	3.9	50.0	Slight variation—still resistant.
33	8		2.0	Later consistently greater than 10.
35	2½	31.25	50.0	
37	4		10.0	Some variation in degree.
38	6		2.0	
39	7		2.0	
41	1		10.0	Later consistently greater than 50.

* The degree of resistance shown may be assumed to mean also "less than" the next highest concentration of amithiozone per cc. used in the test, as described in the text.

of resistance encountered was concerned. The method in which solid medium was used was superior because it gave results more rapidly, with less work for the laboratory staff and with more consistent results. Therefore, this method was used almost exclusively toward the end of the study.

Of the 45 patients, 8 achieved sputum or gastric washings negative for *M.*

tuberculosis on culture during, or at the end of, the course of treatment. Of the 37 whose cultures remained "positive," 15 discharged tubercle bacilli which were consistently drug resistant during the course of treatment. The earliest appearance of drug-resistant bacilli was noted after one month of treatment. Resistant strains in other cases appeared after two to ten months of treatment. Drug-resistant strains of tubercle bacilli appeared at all of the dosage levels which were used. In 8 of these patients there was definite clinical evidence of loss of the therapeutic effect of the drug concomitant with the appearance of drug-resistant bacilli. Some of these patients were treated early in the study, and drug-sensitivity studies showing the persistence of drug-resistant organisms were performed quite some time after cessation of treatment. In one patient (Case 21), drug-resistant organisms appeared after three and one-half months of treatment. Following this, only two positive cultures were obtained; one showed a lesser degree of drug resistance, and the last one (after eight months of treatment) was again drug sensitive. There were no further positive cultures, and the patient was discharged with inactive disease after receiving a two-stage thoracoplasty. There was no clinical evidence of loss of effectiveness of the drug. The data concerning the amithiozone-resistant strains of *M. tuberculosis* are presented in table 4.

DISCUSSION

Two necessary properties of amithiozone must be proved and accepted if it is to survive as a useful adjunct in the chemotherapy of tuberculosis. First, does it have beneficial effect on pulmonary tuberculosis in man? The answer to this is still in doubt in spite of the confident opinions expressed in extreme terms by those who are convinced that it has or has not. Reliable observations seem to have established its beneficial effect on certain mucous membrane lesions. Less certain are its effects on pulmonary disease although there are trustworthy reports which indicate that it exerts favorable action, especially on recent exudative lesions. There is nothing final about its effect on miscellaneous forms of tuberculosis, and it is not recommended for miliary tuberculosis or tuberculous meningitis. Improvement of pulmonary disease was observed in 26 patients in the present series. All lesions had remained stationary or become worse on general supportive treatment and some had no longer improved with streptomycin or para-aminosalicylic acid. It is difficult, if not impossible, to evaluate the relative effect of bed rest and dietary and hygienic therapy. It can be said that the disease had increased or had failed to improve as a result of such treatment up to the time amithiozone was administered.

The second property to be established is whether or not amithiozone can safely be administered in effective dosage. It is admittedly less effective than streptomycin alone or in combination with para-aminosalicylic acid. The writers know of no reliable observations on the relative clinical value of amithiozone versus, or in combination with, para-aminosalicylic acid. Both are less effective than streptomycin. The value of combinations of amithiozone with other drugs having antituberculous activity must await further demonstration and clarifica-

tion. It is, therefore, assumed that streptomycin alone, or in combination with para-aminosalicylic acid, should be used in preference to a less effective drug. There are, however, certain times when another chemotherapeutic agent is to be desired because of a failure, of cessation of tuberculosis to respond to streptomycin with or without para-aminosalicylic acid, because of drug-resistant bacilli, toxicity, or other reasons.

Hepatotoxicity is often encountered with the originally recommended daily dosage of 200 to 300 mg. of amithiozone, or even in smaller dosage of 100 to 150 mg. It occurred in mild or moderate degree in 26, or more than half of the patients treated in the present study. Toxicity seems to be reduced with a decrease in dosage or if the drug is discontinued. Normal values of liver function returned. Hepatic toxicity has been universally observed by all investigators. Falk, Zieve, Tucker, and Hanson (11), in a recent report on hepatic toxicity, observed progressive liver dysfunction in 8 of 14 patients. However, this effect was noted only in patients with evidence of pre-existing hepatocellular dysfunction. Mild anemia and slight reduction in leukocytes showed a suppressive effect on the hemopoietic system. In the present experience, this effect was lost and values returned to normal when the drug was discontinued. Symptomatically, the present writers observed no more unpleasant effects than with other chemotherapeutic agents in use. It is believed that certain patients may justifiably be treated with amithiozone in daily dosage, not to exceed 150 mg., under conditions in which toxic effects can be gauged clinically and by frequent laboratory methods while the patient is under treatment.

SUMMARY AND CONCLUSIONS

The antituberculous activity of amithiozone on experimental tuberculosis in guinea pigs has been demonstrated. Limited, though trustworthy, clinical observations indicate that amithiozone exerts some antituberculous effect on certain tuberculous lesions, including pulmonary, in man. Serious toxic effects resulting from its administration limit its usefulness as a chemotherapeutic adjunct in the treatment of human tuberculosis. When administered in situations in which proper clinical and laboratory observations can be made, it may properly be used for patients who have ceased to improve, or have worsened, under general supportive treatment and other chemotherapeutic agents.

SUMARIO Y CONCLUSIONES

Observaciones Relativas al Efecto de la Amitiozona (Tibiona) en Lesiones Pulmonares Tuberculosas Seleccionadas

Queda demostrado de nuevo el efecto antituberculoso de la amitiozona sobre la tuberculosis experimental del cobayo. Limitadas, aunque fidedignas, observaciones clínicas indican que la amitiozona ejerce algún efecto antituberculoso sobre ciertas lesiones tuberculosas, incluso pulmonares, en el hombre. Graves efectos tóxicos procedentes de su administración limitan la utilidad de la droga como coadyuvante quimioterapéutico en el tratamiento de la tuberculosis humana. Administrada donde cabe llevar a cabo adecuadas observaciones clínicas y de laboratorio, puede emplearse debidamente en los enfermos que han dejado de mejorar, o que han empeorado, unida al tratamiento reconstituyente general y a otros agentes quimioterapéuticos.

RESUME ET CONCLUSIONS

Observations sur l'action de l'amithiozone (Tibione) dans les lésions tuberculeuses pulmonaires sélectionnées

L'action antituberculeuse de l'amithiozone sur la tuberculose expérimentale du cobaye est de nouveau démontrée. Des observations cliniques limitées, mais irrécusables, indiquent que l'amithiozone exerce un certain degré d'action antituberculeuse sur certaines lésions tuberculeuses, y compris les lésions pulmonaires chez l'homme. Des réactions toxiques importantes déterminées par son administration limitent son emploi comme auxiliaire chimiothérapeutique dans le traitement de la tuberculose humaine. Quand ce composé est administré dans des conditions où l'observation clinique et les examens de laboratoire sont possibles, son emploi peut être justifié chez des malades dont l'état a cessé de s'améliorer ou s'est aggravé, malgré un traitement général ou l'administration d'autres agents chimiothérapiques.

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FLUORESCENCE MICROSCOPY IN EXAMINATION OF SMEARS FOR MYCOBACTERIUM TUBERCULOSIS[1]

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INTRODUCTION

The detection of acid-fast bacilli in smears of sputum, pus, and other pathologic material is the most rapid laboratory method available for the presumptive diagnosis of tuberculosis. In many cases the organisms are so dispersed that the discovery of even one or two organisms demands a long and tedious search. With the classical technique of Ziehl-Neelsen, using a one-twelfth–inch oil immersion objective, it has been estimated by Corper and Cohn (1) that in sputum a concentration of at least 100,000 organisms per milliliter is necessary before a positive finding can reasonably be expected.

The Ziehl-Neelsen method stains the bacilli red and they are observed, as a rule, on a blue counterstained background through a one-twelfth–inch oil immersion objective: using a six times magnification eyepiece, a field diameter approximately 0.20 to 0.25 mm. is covered, and the defined depth of focus with a one-twelfth–inch objective (n.a. 1.25) is of the order 0.5 μ only; hence, the total volume in focus at any one time is small (approximately 2 by 10^{-6} cu. mm.). The lack of contrast between the red-stained bacilli and the blue background militates against success in indentifying bacilli slightly out of focus. It has been found, however, that the method of fluorescence microscopy has a number of advantages in scanning stained smears for acid-fast bacilli. The advantages are as follows:

1. The bright fluorescent bacilli are much more obvious owing to increased contrast against the dark background; they can, in fact, be detected by their luminous halos when somewhat out of focus.
2. The fatigue entailed in examining films is reduced by the increase in contrast.
3. The bacilli can be recognized at a lower magnification when stained by this method; hence, not only can a larger area (diameter approximately 0.5 mm.) be covered in each field, but, by using an objective of smaller aperture, a considerably greater depth of focus (both defined and effective) is achieved. The defined depth of focus of a one-sixth–inch objective (n.a. 0.5) is approximately 1μ. Thus, the *volume* of material in defined focus in a single field, namely, 2 by 10^{-4} cu. mm., is ten times greater with the fluorescence technique than with Ziehl-Neelsen.
4. The time required to search a smear effectively is much reduced. It is the writer's practice to cover 100 fields in routine examinations; this is done in approximately two minutes.
5. To those who suffer from red-green color blindness, which is not uncommon, the Ziehl-Neelsen method has an additional shortcoming which is not encountered if fluorescence microscopy is used.

[1] From the Public Health Laboratory, Bacteriology Department, University of Melbourne, Australia.

The application of fluorescence microscopy to the detection of acid-fast bacilli, particularly of *M. tuberculosis*, was introduced by Hagemann in 1938 (2).

The principle of the method is that the bacilli are stained with the fluorochrome, auramine, to which they are acid and alcohol fast; the remainder of the material is decolorized with acid alcohol. Auramine has the property of converting the light at the violet end of the spectrum to rays of a longer wave length in the yellow wave-band. By inserting between the light source and the object a blue filter, which cuts out the longer wave-lengths including yellow, the stained bacilli are caused to fluoresce. When a yellow filter is incorporated in the eyepiece, all background blue light is blocked, so the stained bacilli appear yellow on a dark field. If the source gives a high intensity of the fluoresceogenic wave bands, the fluorochrome-stained objects will be seen in high contrast; a magnification of two to three hundred times is quite sufficient to recognize acid-fast bacilli.

The method was developed and publicized by Lempert (3), Hughes (4), and others, who acclaimed its advantages.

After extensive comparative trials against the Ziehl-Neelsen technique, fluorescence microscopy was adopted for routine examination for acid-fast bacilli in this laboratory as far back as 1946; during the ensuing years it was found to give the following advantages:

1. Staining of films was quicker and cleaner.
2. Examination was quicker, and the comparative tests showed that it actually gave a larger number of positive findings in culture-positive specimens.
3. Examination was less fatiguing to the examiner, owing to the higher contrast of the staining method and the wider coverage per field.

The use of quartz lenses and aluminum mirrors originally employed by Hagemann was found to be unnecessary; thereafter, the main shortcoming of the method was the repeated cost of replacing the expensive lamp which was found to be the most convenient source of ultraviolet light. It has now been shown by Matthaei (5) that the fluorescence of auramine-stained tubercle bacilli can be produced by blue light of wave length up to 500 $\mu\mu$. For monocular work, a sufficient intensity of this radiation can be obtained from an ordinary 12-volt, 100-watt projection lamp, *provided it is working at its maximum output.*

Despite its potential benefits to a busy laboratory, fluorescence microscopy for detection of *M. tuberculosis* has received a considerable amount of adverse criticism from both sides of the Atlantic, and more than one writer has expressed the view that little is to be gained from its adoption in place of the classical Ziehl-Neelsen method or modifications thereof. Such divergence from the opinion of workers in this laboratory has prompted the present writer to examine the techniques employed by the critics, and to compare them with the routine procedure used in this laboratory from the standpoint of practical as well as theoretical considerations.

The use of a long focus one-seventh-inch oil immersion lens with Ziehl-Neelsen technique has been advocated in some quarters in order to cover a

greater field area. This certainly has such an advantage over the one-twelfth–inch objective, both in the area and depth of the field, but the benefit of a further increase is achieved by using a one-sixth–inch dry objective. A modified Ziehl-Neelsen method using a one-seventh–inch oil immersion objective, no counterstain, and a Wratten H45 substage filter described by Briggs and Jennison (6) was tried. Although the carbol-fuchsin–stained organisms can be clearly seen, they cannot always be distinguished from unstained debris with sufficient certainty to compete even with the classical method. As a result of his critical re-examination of the fluorescence technique from the optical standpoint, Matthaei (5) has concluded that the use of a one-sixth–inch objective with an eyepiece, giving a magnification of 6 to 10 diameters, and the standard condenser (n.a. 1.2) with the diaphragm open, together with a 100-watt projection lamp and suitable filters, gives the optimal results in a routine laboratory, *provided always that proper attention is paid to the details of the setup which he describes.*

The method to be described in the present report is applicable, using the microscope available in any pathologic laboratory. The only extra accessories required are the filters and the source of light, all of which are available at moderate cost. The lamp itself is extremely good for routine microscopy.

MATERIALS AND METHODS

Slide Preparation for Fluorescence Microscopy

Smear preparation: Smears are fixed at 70° to 75°C. on a hot plate for five to ten minutes. Excessive heating impairs staining properties, but this fixation temperature (dry heat) does not kill *M. tuberculosis* in sputum. When culture of the sputum is not required, however, the specimens may be rendered safe by heating in a water bath at 80°C. for fifteen minutes *before* preparing the slide. (If not killed by heat, the organisms will not survive the acid alcohol washing, but preheating is safer.)

Stain:

Auramine O.S.[2]	3.0 gm.
Rhodamine B.S.[2]	1.5 gm.
Glycerol (analytical reagent)	150 ml.
Phenol crystals liquefied at 50°C.	20 ml.
Distilled water	100 ml.

The solution is lightly agitated and brought to boiling temperature to dissolve the stain. The mixture keeps for several weeks if well stoppered.

Staining technique: Stain, conveniently heated in a 100 ml. drop bottle to 50°–60°C., is poured onto fixed smears, left without further heating for ten minutes, and then washed off with tap water. The preparation is then decolorized with 1 to 2 per cent hydrochloric acid (V/V) in 70 per cent ethanol, using three changes: the first, for one minute; and the second and third, for two minutes each (this will kill any surviving organisms). The preparation is washed with tap water and drained and dried on racks on a hotplate (slide temperature, approximately 50° to 60°C.) N.B. If kept for a few days the organisms lose their brilliance and may need to be re-stained.

Copper sulfate filter: An 8-ounce flat medicine bottle of white glass (outside measurements 1 by 2½ by 7 inches), inside clearance approximately 2.5 cm., is nearly filled with the following stock solution:

[2] Obtained from Imperial Chemical Industries, Ltd.

$CuSO_4 5H_2O$	8.33 gm.
Ammonium hydroxide (d = 0.880)	100 ml.
Distilled water	166 ml.

The concentration of ammoniacal copper sulfate solution may need some adjustment owing to variability in the light source, in the dimensions of the bottle, et cetera. To produce the optimum results, a freshly stained "positive" smear should be examined and the final concentration of the solution in the filter bottle varied to give maximum contrast of golden-yellow bacilli against a dark olive-green background. At the strength prepared, the background will be intensely black. Gradual addition of distilled water lightens the background to give the required contrast; further dilution will alter the color of the background to a luminous blue-green. Addition of a little stock solution of ammoniacal copper sulfate will bring back the desired background.

Examination: Examination of the preparation should be conducted in a dark cabinet or darkened room. A drop of glycerin or liquid paraffin is placed on the condenser, the slide is placed on the stage, and the condenser is raised so that the liquid seals the condenser to the slide; this effects a considerable increase in the light reaching the object. With a light source of high intensity, it may be practicable to dispense with the somewhat messy liquid seal between the condenser and slide. (Cedar oil is unsuitable since it has a native fluorescence, but water will do although it lacks the desired viscosity.) A dry coverslip is placed over the film, if an ordinary one-sixth-inch objective is used, and the objective is lowered. The object is then brought into focus; if no fluorescent bacilli are present, the details in the background can just be made out as the slide is moved and its surface is brought into focus. *After each examination the coverslip should be discarded.* The use of a coverslip may be obviated by using a specially compensated object lens, such as the type used in metallurgical work. For routine work, after completing the examination of one slide, it is more convenient to swing the objective to clear the microscope table than to raise the objective. When the new slide is in place, the objective can be returned to position and the slide will be approximately in focus.

Experimental procedure: Duplicate smears were made from a specimen of sputum which contained a fair number of tubercle bacilli. One preparation was stained by the classical Ziehl-Neelsen method, and the other by the auramine-rhodamine method in current use in this laboratory. The following equipment, shown in figure 1, was employed in these experiments:

Monocular microscope
Eyepiece of six times magnification
Abbe condenser, n.a. 1.2
Filament lamp (projection type), 12 volts 100-watt, with a 300 ml. Florence flask full of water as a condenser fitted to the lamp housing (figure 1).

The whole equipment was so placed as to project the image of the transversely placed filament into the plane of the microscope condenser diaphragm with the minimum loss of light. Examination was conducted in a dark cabinet. Only the method of staining with illumination and the type of objective were varied.

With the Ziehl-Neelsen technique, the substage condenser iris diaphragm was closed sufficiently to give optimal definition. Two objectives were compared as follows:

1. Objective one-twelfth-inch oil immersion (n.a. 1.2), magnification times 90.

2. Objective one-seventh-inch oil immersion (n.a. 1.05), magnification times 60.

With the fluorescence technique, a copper sulfate filter (see above) was placed between the Florence flask and the mirror of the microscope to cut out all wave lengths over 500μμ. A Wratten gelatin filter cut to size was inserted in the eyepiece, resting on the field lens. The condenser diaphragm was fully opened and a drop of glycerin placed on the upper surface of the condenser. The smear was stained as described above, and contact was established between the under surface of the slide and the glycerin on the upper surface of the condenser.

FIG. 1. Simple arrangement for observation of fluorescence projection filament lamp in tin container, spherical Florence flask as lamp condenser, and medicine bottle containing ammoniacal copper sulfate solution as blue filter.

1. Objective one-sixth-inch (ordinary achromatic high power) n.a. 0.65, magnification times 40; a dry coverslip was placed over the smear.

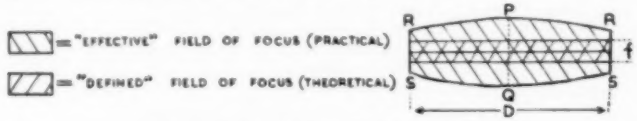
2. Objective one-sixth-inch metallurgical, n.a. 0.65, magnification times 40.

Since this is suitably corrected, a coverslip is unnecessary. (This lens is used in routine work in this laboratory since it obviates the use of a fresh coverslip for each smear, but system (*1*) is perfectly satisfactory where a limited number of smears are examined.)

The defined depth of focus can be determined theoretically for any optical system; the area of the field depends on the eyepiece (which was kept constant) and on the objective; to compare different staining methods the following practice was employed:

A single organism was placed in the center of the field and carefully focused; the microscope was adjusted until the organism disappeared out of focus; the objective was then slowly lowered until the selected bacillus was judged to be just so discernible that it might be noticed in routine scanning; the "reading" was noted as the "upper limit of discerni-

bility." The organism was next focused as accurately as possible and a second "reading" was made. Finally, after lowering out of focus, the "lower limit of discernibility" was gauged by raising the objective. The distance between upper and lower limits gives the "effective focal depth" at the center of the field (PQ in diagram).



DIAGRAMATIC SECTION OF FIELD
IN WHICH AN ORGANISM CAN BE PERCEIVED

TABLE 1

COMPARISON BETWEEN ZIEHL-NEELSEN AND FLUORESCENCE MICROSCOPY METHODS FOR THE DETECTION OF ACID-FAST BACILLI

STAINING METHOD	OBJECTIVE	THEORETICAL			PRACTICAL			
		Diameter of Field D	Defined Depth of Focus f	Approximate volume of defined field $\frac{\pi D^2}{4} \times \frac{f}{10^3}$	Diameter of Field D	PQ*	RS*	Volume V of Field = $\frac{\pi D^2}{4} \times \frac{(PQ + RS)}{2}$
		mm.	μ	cu. mm.	mm.	μ	μ	
(a) Ziehl-Neelsen	(1) $\frac{1}{12}$-inch oil n.a. 1.25 ×90	c 0.25	0.4	2×10^{-5}	0.24	8.8	0	2×10^{-4} cu. mm. approximately
	(2) $\frac{1}{7}$-inch oil n.a. 1.05 ×60	c 0.35	0.6	6×10^{-5}	0.37	10.4	0	5×10^{-4} cu. mm. approximately
(b) Auramine-rhodamine fluorescence	$\frac{1}{6}$-inch standard dry	c 0.5	1.0	2×10^{-4}	0.56	19.6	18.6	5×10^{-3} cu. mm. approximately
	$\frac{1}{6}$-inch metallurgical	c 0.5	1.0	2×10^{-4}	0.44	24.4	26.6	3.9×10^{-3} cu. mm. approximately

* Average of 10 separate readings.

The same bacillus was now moved to the extreme edge of the field and similar observations were made of the limits and hence the extent of the focal depth at the periphery (RS in diagram). The readings for optimal focus were found to be approximately midway between these limits; the determination of the limits was by no means exact but, in each case, readings on 10 separate organisms were made and the average values of PQ and RS were determined.

The diameter of the field was measured with the aid of a micrometer slide.

The effective volume V of the field can be approximately computed from the following formula: $V = \frac{\pi D^2}{4} \times \frac{PQ + RS}{2}$.

RESULTS

As will be seen from table 1, the effective field volume is greatest with the fluorescence technique since, owing to their brightness, the bacilli are recognizable at

lower magnification than is possible with the Ziehl-Neelsen method. Again, due to spherical aberration in the standard optical system, it is difficult or well-nigh impossible with the Ziehl-Neelsen method to detect bacilli toward the periphery of the field. With fluorescence microscopy, however, the out-of-focus images of the stained organisms can be detected even at the extreme edges of the field by virtue of their striking contrast and brightness and their color, rather than by their shape. Once such a particle is seen, it can be centered and focused for proper examination. With the Ziehl-Neelsen method, use of a substage filter, although it may increase the ability to see the bacilli, is not helpful in diagnosis, since the bacilli lose their distinctive color and can be mistaken for an item of debris or a scratch on the slide, which appears as a dark line.

One interesting fact that appeared, however, in collecting the figures, is that there seems to be a considerable variation in depth of staining by both methods. This may be due in part to variation in the accessibility of a particular organism to the stain or to the light owing to irregularities in the smear, and also, perhaps, to difference in the quality of the individual bacilli.

TABLE 2

COMPARATIVE RESULTS ON 1,098 SPUTUM SPECIMENS IN A SERIES SUBMITTED FOR ROUTINE EXAMINATION

METHOD	POSITIVE	NEGATIVE	TOTAL	PER CENT POSITIVE
Ziehl-Neelsen $\frac{1}{12}$-inch immersion	146	952	1,098	13.3
Fluorescence microscopy $\frac{1}{6}$-inch	158	940	1,098	14.4

As far as possible, a single organism was selected for each reading, and one whose axis appeared to lie in the plane of the field. There is little doubt that, with any method of staining, two or more bacilli lying in contact show up in higher contrast than one, and an oblique posture patently increases the depth of perceptibility.

From the tabulated results it is evident that, whereas the Ziehl-Neelsen technique with one-seventh–inch objective gives a field volume of approximately 6 by 10^{-4} cu. mm. and with the one-twelfth–inch objective of 2 by 10^{-5} cu mm., the fluorescence microscopy field volume lies in the region of 5 by 10^{-3} cu. mm., an increase in the order of twenty-fold.

In the foregoing experiments, it is probable that the effective field of focus with both methods may be slightly greater than it would be in practice, since the position in the field of the particular organism under examination was accurately known as it was brought into focus. As already discussed, the fluorescence image is of higher contrast, and is, therefore, more arresting to the searching eye than the Ziehl-Neelsen image. Hence, from the knowledge just where to look for the appearing image, the Ziehl-Neelsen method may in the experiment derive some advantage, relative to the fluorescence technique, which it would not enjoy in practice.

The two methods were applied to a series of 1,098 specimens of sputum submitted for routine examination. Smears were made in duplicate and one of each pair was stained and examined by Ziehl-Neelsen and the other by the fluorescence technique for the appropriate time by one of a pair of observers. Halfway through the series the two examiners exchanged methods. Of these, 146 were found to be positive by both methods, and an additional 12, making a total of 158, were positive by the fluorescence method (table 2).

COMMENT

The present writer is completely convinced that the fluorescence method, properly applied, is superior to the Ziehl-Neelsen method, and this work was undertaken only because of the publications of others who seem to be unacquainted with the possibilities of the former technique. Without proper attention to such details as the preparation of stained smears, the intensity of the light reaching the organisms, and its wave length (the conservation of the rays emitted by the source and the adjustment of the blue filter), with the use of a dark cabinet, the fluorescence method could readily fall into disrepute, and a very valuable diagnostic tool be consigned to the limbo of mere academic interest where it surely does not belong.

SUMMARY

Comparison on both theoretical and experimental grounds is drawn between the classical Ziehl-Neelsen and the fluorescence microscopy methods, using the appropriate optical systems, for the detection of acid-fast bacilli in pathologic material.

It is concluded that the fluorescence microscopy method is preferable since the organisms can be detected in a field of considerably greater volume than is covered using the classical method.

The fluorescence microscopy technique as employed in this laboratory is briefly described.

SUMARIO

La Microscopía Fluorescente en el Examen de Frotes en Busca del Mycobacterium Tuberculosis

La comparación aquí trazada sobre base tanto teórica cuanto experimental es entre la clásica técnica de Ziehl-Neelsen y la microscopía fluorescente, usando sistemas ópticos adecuados, para el descubrimiento de bacilos ácidorresistentes en ejemplares pathológicos.

Dedúce que la técnica de la microscopía fluorescente es preferible, dado que permite descubrir los microbios en un campo mucho más voluminoso que el abarcado cuando se emplea el método clásico.

Describese sucintamente la técnica de la microscopía fluorescente, tal como se emplea en el laboratorio del A.

RESUME

La microscopie en fluorescence dans l'examen des frottis pour la recherche du Mycobactérium Tuberculosis

Une comparison, tant d'ordre théorique qu'expérimental, est faite entre les méthodes classiques Ziehl-Neelsen et la microscopie en fluorescence, à l'aide de systèmes optiques appropriés, pour la détection des bacilles acido résistants dans les matières pathologiques.

En conclusion, la méthode de microscopie en fluorescence est considérée préférable, car elle permet de déceler les microorganismes dans un champ d'étendue beaucoup plus grande que celui couvert par la méthode classique. La technique de microscopie en fluorescence, telle qu'elle est employée dans ce laboratoire, est brièvement décrite.

Acknowledgments

Much of the work which established this fluorescence technique was carried out by my predecessor, Dr. A. A. Ferris, and by my colleagues in this laboratory, particularly by Mr. L. J. Swaby; to them and to Professor S. D. Rubbo, I am grateful for much helpful advice. I am also beholden to Mr. E. Matthaei for his ready aid and constructive criticism in the comparison of the methods under review, and for the photograph of the system used.

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ANTITUBERCULOUS ACTIVITY IN MICE OF TRITON A-20, A NONIONIC ALKYL-ARYL POLYETHER ALCOHOL, USED ALONE AND IN COMBINATION WITH DIHYDROSTREPTOMYCIN[1,2]

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With the technical assistance of THOMAS H. WOMBOLT

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INTRODUCTION

While studying the effect of cortisone on the course of tuberculosis in mice, it was observed that Tween 80, a component of the cortisone vehicle, slightly accelerated the progress of the infection. In further investigation of this effect, Triton A-20, a nonionic alkyl-aryl polyether alcohol, was selected as a control because it possesses low tuberculostatic activity *in vitro* (1), is tolerated by mice, and possesses ether linkages which are not split by enzymes known to be present in man. Surprisingly, Triton A-20 was found to possess chemotherapeutic activity. The chemotherapeutic activity of Triton A-20 has now been reported by Cornforth, Hart, Rees, and Stock (2). They have prepared a series of similar substances of better defined composition, not possessing greater efficacy than the original but possibly less toxic. The present report confirms the findings of Cornforth and associates and, in addition, shows that Triton A-20 acts synergistically with dihydrostreptomycin (DHSM).

METHODS AND MATERIALS

Barckmann IS-32 white Swiss mice, weighing 15 to 17 gm., were infected intravenously with 0.25 ml. amounts of suspension of *Mycobacterium tuberculosis*, human type, strain H37Rv, in Dubos Tween-albumin medium. The cultures were adjusted to permit 70 per cent transmission of light at 620 mμ with a Coleman 6A spectrophotometer. A subculture producing rapidly fatal infection (average survival time, twenty to twenty-five days) and one producing slowly progressive infection (average survival time, more than fifty days) were used. Treatment was begun twenty-four hours after infection. Triton A-20 (lot WR1339)[3] and dihydrostreptomycin sulfate were dissolved in distilled water at concentrations containing the desired daily dose per mouse in 0.25 ml. amounts of solution. The compounds were administered subcutaneously once daily five days per week.

Tests with the rapidly fatal infection were continued until at least half of the infected controls were dead, and those with the slowly progressive infection were terminated forty-five days after infection. The lungs of all mice dying during the course of the experiments, as well as those mice surviving at the end of the test period, were preserved in 10 per cent formalin. After a minimal fixation period of two days, the lungs were examined for the degree of gross tuberculous involvement. A score of 1 plus represented minimal involvement (10 per cent of lung area); 2 plus, moderate involvement (10 to 25 per cent of lung area); 3 plus, moderate to severe involvement (25 to 50 per cent of lung area); and 4 plus,

[1] From the Merck Institute for Therapeutic Research, Rahway, New Jersey.

[2] Presented at the Eleventh Conference on The Chemotherapy of Tuberculosis, Veterans Administration, St. Louis, Missouri, January 17–20, 1952.

[3] Supplied by Rohm and Haas.

severe involvement (more than 50 per cent of lung area). A 2 times magnifying glass facilitated the examination.

EXPERIMENTAL OBSERVATIONS

Triton A-20 used alone against a rapidly fatal infection: In a representative experiment, groups of 8 mice each were infected intravenously and treated subcutaneously once daily with 0.6, 1.2, 2.5, and 6.3 mg. per mouse. For positive controls, dihydrostreptomycin was administered to comparable groups of mice at doses of 0.25, 0.5, 1.0, and 2.0 mg. per mouse per day. The test was terminated nineteen days after infection, at which time 4 of 8 infected controls were dead. The results are shown in figure 1. Triton A-20 was ineffective at 0.6 mg., mod-

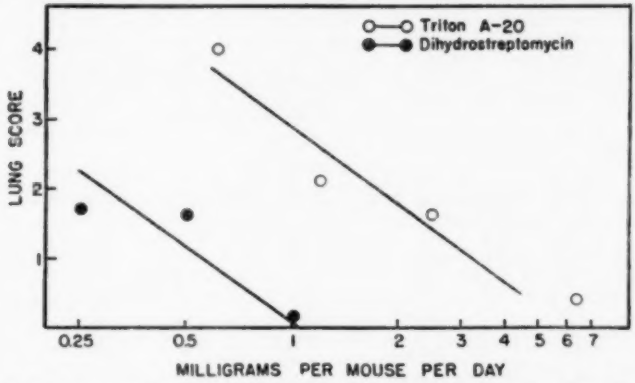

FIG. 1. The comparative efficacy of Triton A-20 and dihydrostreptomycin. Gross lung involvement of mice infected with *M. tuberculosis*, human type, and treated with Triton A-20 or dihydrostreptomycin. Each point represents the average lung score for a group of 8 mice.

erately effective at 1.2 and 2.5 mg., and highly effective at 6.3 mg. Dihydrostreptomycin was moderately effective at 0.25 and 0.5 mg., and highly effective at 1 and 2 mg. A comparison of the curves suggested that Triton A-20 was approximately one-sixth as active as dihydrostreptomycin.

The combined effect of Triton A-20 and dihydrostreptomycin against a prolonged infection: Twenty-five groups of 8 mice each were treated with combinations of Triton A-20 and dihydrostreptomycin, or either agent alone, as shown in table 1. Treatment was administered for the entire test period. This table summarizes the gross pathologic lung involvement observed forty-five days after infection. Four levels of Triton A-20 were used ranging from 0.625 to 5.0 mg., increasing by twofold amounts, and dihydrostreptomycin was used similarly in doses from 0.125 to 1.0 mg. All combinations of doses within these two ranges were tested.

The infected controls had a score of 4.0. Under the conditions of prolonged infection, the highest dose of dihydrostreptomycin reduced the score to 2.1, and

the highest dose of Triton A-20 produced the same reduction in lung involvement. The addition of 0.625 mg. of Triton A-20 to dihydrostreptomycin, a dose at which Triton A-20 alone was ineffective, enhanced the activity of dihydrostreptomycin at doses of 0.25 mg. and higher. Triton A-20 alone was also ineffective at 1.25 mg., but this dose raised the efficacy of dihydrostreptomycin at levels of 0.25 mg. and higher. The addition of 2.5 mg. of Triton A-20 increased the activity of all levels of dihydrostreptomycin; and with 5.0 mg. of Triton A-20, increased activity was observed for dihydrostreptomycin at 0.25 mg. and ascending doses. Further inspection of the data showed that a series of nine combinations produced efficacy superior to that attained with the maximal amounts of either drug alone; these are emphasized in the table by use of heavier type. The 0.625 mg. dose of Triton A-20 produced this effect with the highest dose of

TABLE 1

THE COMBINED ACTIVITY OF TRITON A-20 AND DIHYDROSTREPTOMYCIN

DHSM MG. PER MOUSE PER DAY	TRITON A-20—MG. PER MOUSE PER DAY									
	0		0.625		1.25		2.5		5.0	
	Gr. lg.* involv.	No.surv.† Total	Gr. lg. involv.	No. surv. Total	Gr. lg. involv.	No. surv. Total	Gr. lg. involv.	No. surv. Total	Gr. lg. involv.	No. surv. Total
0	4.0	3/8	4.0	3/8	4.0	2/8	3.4	8/8	2.1	8/8
0.125	3.2	6/8	3.4	7/8	3.5	8/8	1.9	7/7	2.0	8/8
0.25	3.3	5/8	2.8	8/8	2.4	8/8	**0.9**	8/8	**0.4**	8/8
0.5	3.2	7/8	2.7	8/8	**1.1**	8/8	**0.4**	8/8	**1.4**	8/8
1.0	2.1	6/7	**0.9**	8/8	**0.8**	7/7	**0.6**	8/8	**0.5**	7/7

* Gross lung involvement.

† Number of surviving/total.

dihydrostreptomycin, and the increasing doses of Triton A-20 similarly enhanced the activity of diminishing doses of dihydrostreptomycin.

Toxicity: The acute toxicity studies in mice show that single doses of 12.5 and 25.0 mg. per 20-gram mouse were not lethal. Larger doses, ranging from 50.0 to 75.0 mg. per 20-gram mouse, were lethal. Animals receiving the 25.0 mg. dose level became depressed within thirty minutes after the injection, but subsequently recovered (3).

SUMMARY

Triton A-20, a nonionic alkyl-aryl polyether alcohol, was tested for antituberculous activity in the mouse, both alone and in combination with dihydrostreptomycin. The agent was administered parenterally, and efficacy was determined by evaluation of gross pathologic involvement in the lungs.

Triton A-20 alone was approximately one-sixth as active as dihydrostreptomycin. When used together with the latter drug in a five factorial experiment, Triton A-20 produced a synergistic effect. As examples of this synergistic effect: 1.25 mg. of Triton A-20 combined with 0.5 mg. of dihydrostreptomycin were

more effective than 5.0 mg. of Triton A-20 or 1.0 mg. of dihydrostreptomycin alone; 2.5 mg. of Triton A-20 combined with 0.25 mg. of dihydrostreptomycin were more effective than 5.0 mg. of Triton A-20 or 1.0 mg. of dihydrostreptomycin alone.

SUMARIO

Actividad Antituberculosa en los Ratones del Tritón A-20, Alcohol Aniónico Alquil-Aril Polietéreo, Usado Ya Solo o Combinado don la Dihidroestreptomicina

El Tritón A-20, alcohol aniónico alquil-aril polietéreo, fué comprobado en cuanto a actividad antituberculosa en el ratón, tanto solo como combinado con la dihidroestreptomicina. Dicho agente fué administrado parentéricamente, determinándose su eficacia por la justipreciación de la invasión macropatológica en los pulmones.

El Tritón A-20 solo mostró aproximadamente la sexta parte de la actividad de la dihidroestreptomicina. Usado conjuntamente con la última en un experimento de cinco factores, produjo efecto sinérgico. Ejemplos de este efecto sinérgico son: 1.25 mg. de Tritón A-20 unidos a 0.5 mg. de dihidroestreptomicina fueron más eficaces que 5.0 mg. de Tritón A-20 o que 1.0 mg. de dihidroestreptomicina solos; 2.5 mg. de Tritón A-20 combinados con 0.25 mg. de dihidroestreptomicina fueron más eficaces que 5.0 mg. de Tritón A-20 o que 1.0 mg. de dihidroestreptomicina solos.

RESUME

Activité antituberculeuse, chez la souris, du Triton A-20, un alcool polyéther alcalyl-aryl non ionique, employé exclusivement ou en association avec la dihydrostreptomycine

Le Triton A-20, un alcool polyéther alcalyl-aryl non ionique a été expérimenté seul, ou en association avec la dihydrostreptomycine, pour évaluer son action antituberculeuse chez la souris. L'efficacité de cet agent, administré par voie parentérale, a été déterminée par l'évaluation des lésions macroscopiques dans le poumon.

L'activité du Triton A-20 seul était approximativement du sixième de celle de la dihydrostreptomycine. Utilisé avec la streptomycine au cours d'une expérimentation comportant cinq facteurs, le Triton A-20 produisait un effet de synergie. A titre d'exemple de cette action synergique: 1,25 mg. de Triton A-20 associé à 0,5 mg. de dihydrostreptomycine était plus efficace que 5 mg. de Triton A-20 ou 1 mg. de dihydrostreptomycine administrés isolément; 2,5 mg. de Triton A-20 associé à 0,25 mg. de dihydrostreptomycine était plus efficace que 5 mg. de Triton A-20 ou 1 mg. de dihydrostreptomycine administrés isolément.

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ENZYMATIC REACTIONS OF MYCOBACTERIUM TUBERCULOSIS AND THE ACTION OF STREPTOMYCIN[1]

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INTRODUCTION

Although common enzymes of other microorganisms and cells are also present in the tubercle bacillus, some features of their action and the products recovered from growing tubercle bacilli either signify an individuality of these enzymes or a difference between those commonly identified by the same type and name. Thus, the recognition of the presence of lipases (1), various proteolytic enzymes, nucleases, ureases, reductases, and oxidases qualitatively is of long standing. It is a well-established fact that the tubercle bacillus, through its enzymes, forms tuberculin, a protein, but is incapable of carrying this process of enzymatic action to a lower molecular stage (2) even though pepsin-like and trypsin-like enzymes are attributed to this microorganism (3). At an early period tubercle bacilli were also demonstrated to possess catalases (4), yet even today a complete study of the enzymes of the tubercle bacillus is lacking. Except for a few examples, the significance of their action is not disclosed, probably because of the difficulties encountered with a slowly evolving and markedly resistant organism and its reactions to profound physical and chemical changes, as well as possession of a wide latitude of growth ability. Although marked progress has been made in isolating and defining some enzymes and certain methods for measuring enzyme activity are relatively easy to perform, many are ill defined and difficult to interpret. Although enzymes are viewed primarily as accelerators, probably the enzyme actually initiates the reaction (5). With the advent of antibiotics and the interest in finding the explanation of their action on certain bacterial infections, the enzymes of these microorganisms take on added significance as a possible source of such action. In addition, the still undisclosed mechanism of virulence, particularly of the tubercle bacillus, again calls attention to possible enzymatic differences.

In 1949, Oginsky, Smith, and Umbreit (6) reported that streptomycin acted upon *E. coli* by inhibiting an oxidative reaction in susceptible strains. They believed that the reaction was apparently the "oxalacetate-pyruvate" condensation and, when it was inhibited, a variety of substances were prevented from entering the terminal respiration system that resembles the citric acid cycle. They further postulated that the ability to grow in the presence of streptomycin depends upon the development of unknown reactions permitting the cell to dispense with this condensation (7). In a later article, however, Umbreit (8) notes that: "Streptomycin's action against the tubercle organism is perhaps a special case. In these bacteria, it has so far been impossible to demonstrate an oxalacetate-pyruvate reaction."

[1] From the Research Department, National Jewish Hospital at Denver, and the University of Colorado School of Medicine.

Because of the uncertainty of the foregoing and its apparent parallel to the cytochrome reaction, indicated by the methylene blue reduction reaction, it appeared advisable to use the methylene blue reaction quantitatively with tubercle bacilli, particularly since this might also be used as an index of the terminal respiration process (9) and since taking any part of the cytochrome reaction should reveal a change in the entire functional relationship (10). Reasoning on the basis of the oxidation enzyme action, Fitzgerald, Bernheim, and Fitzgerald (11) believe that the oxidation of benzoic acid and its use as the sole carbon source which streptomycin inhibits (as an effect on the benzoic acid oxidase) points to an adaptive enzyme process in certain *Mycobacteria*, the formation of which may be the significant factor in its action. More recently Zeller, Owen, and Karlson (12) claimed that diamine-oxidase of streptomycin-sensitive strains of *M. smegmatis* is inhibited by small concentrations of streptomycin and dihydrostreptomycin, while much larger concentrations are required for streptomycin-resistant strains in order to obtain the same effect.

OBSERVATIONS

Diamine-Oxidase (Histaminase)

Since it was suggested that streptomycin acted through the mechanism of diamine-oxidase (histaminase) and it appeared desirable to determine the presence of such reaction with the tubercle bacillus, it seemed logical, according to Sumner and Somers (13), that any one of 13 diamines should be suitable for test. Since histamine was one of these and had been studied carefully (14), it was used in the following study with a sensitive and resistant human tubercle bacillus of the same strain and also a resistant and sensitive acid-fast saprophyte. The results of these observations are recorded in table 1.

From the results in table 1 it appears that human tubercle bacilli are incapable of destroying histamine base in a buffer phosphate solution at pH 7.3 within five days at 37°C., and that such inability to alter the toxic reaction of histamine is not changed appreciably by the addition of 1 mg. (1,000 units) of streptomycin. These observations do not support the view that a diamine-oxidase (histaminase) is present in an appreciable amount in active form in human tubercle bacilli or that diamine-oxidase (histaminase) is at the basis of the retarding action of streptomycin on human tubercle bacilli. Likewise, no evidence of diamine-oxidase (histaminase) was disclosed in the acid-fast saprophyte tested which is also retarded by streptomycin in the nonresistant form.

Dehydrogenase (Anaerobic) Methylene Blue Reduction

Under careful working conditions, methylene blue can be used as an indicator (acceptor) of hydrogen which comes from an oxidized substrate in the complicated mechanism of the action of dehydrogenases. Tubercle bacilli, like many other microorganisms, can reduce methylene blue enzymatically under certain conditions, and this is claimed by some to vary with the bacilli used (15). In the present studies of this enzyme in regard to the tubercle bacillus, the interest was not only on the bacilli themselves but also on the changes which occurred under certain conditions in the same strain and under the influence of certain retardants, such as streptomycin, dihydrostreptomycin, para-

TABLE 1

THE EFFECT OF A STREPTOMYCIN-SENSITIVE AND STREPTOMYCIN-RESISTANT HUMAN TUBERCLE BACILLUS AND AN ACID-FAST SAPROPHYTE ON HISTAMINE (DIAMINE-OXIDASE) REACTION*

TEST IN PHOSPHATE BUFFER pH 7.3 PER CC. OF MIXTURE	TIME OF CONTACT WITH HISTAMINE 5 MG. PER CC.	RESULTS OF GUINEA PIG INOCULATION	
		Intravenous Injection of 1:10 Dilution (1 cc.)†	Intraperitoneal Injection of 1 cc. Solution †
Control—histamine‡ (base) 5 mg. per cc.		Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin-sensitive human tubercle bacilli 500 mg.§	6 hours 24 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin-sensitive human tubercle bacilli 500 mg. + 1 mg. streptomycin	6 hours 24 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin-resistant human tubercle bacilli 500 mg.	6 hours 24 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin-resistant human tubercle bacilli 500 mg. + 1 mg. streptomycin	6 hours 24 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin - sensitive acid-fast saprophyte 250 mg.‖	4 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin - sensitive acid-fast saprophyte 250 mg. + 1 mg. streptomycin	4 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin - resistant acid-fast saprophyte 250 mg.	4 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes
Streptomycin - resistant acid-fast saprophyte 250 mg. + 1 mg. streptomycin	4 hours 5 days	Lethal within 3 minutes	Lethal within 3 minutes

* This tabulation is a composite of triplicate tests performed at 37°C.

† Minimal lethal dose.

‡ As control, a standard histaminase was used as per reference 14.

§ Culture of tubercle bacilli was on Wong-Weinzirl synthetic nonprotein medium after four weeks at 37°C.

‖ Culture of acid-fast saprophyte (Day) was on Wong-Weinzirl medium after one week at 37°C.

aminosalicylic acid, and the effects induced by streptomycin resistance. In addition, the studies included the effects of acetone extraction for the purpose of preparing stable enzyme products. The results of these studies are recorded in tables 2 and 3.

The data in table 2 indicate that there is no difference in the quantitative reduction of methylene blue under anaerobic conditions between streptomycin-sensitive and streptomycin-resistant human tubercle bacilli. The data further

TABLE 2

THE EFFECT OF STREPTOMYCIN ON REDUCTION OF METHYLENE BLUE BY SENSITIVE AND RESISTANT HUMAN TUBERCLE BACILLI*

TYPE OF TUBERCLE BACILLI	PER CENT REDUCTION AFTER 4 HOURS†		
	Control	1 mg. Streptomycin per cc.	1 mg. Dihydrostreptomycin per cc.
Viable bacilli—sensitive			
10 mg. (moist) per cc.	100‡	100	100
5 mg. (moist) per cc.	90	85	85
1 mg. (moist) per cc.	15	15	15
0.1 mg. (moist) per cc.	10		
Viable bacilli—resistant			
10 mg. (moist) per cc.	100	100	100
5 mg. (moist) per cc.	95	95	100
Acetone extracted bacilli dry bacilli extracted 24 hours 57°C. 50 mg. (dry) per cc.§	100	100	100

* This tabulation is a composite of triplicate tests performed at 37°C.

† All methylene blue reductions were carried out under anaerobic conditions in a Thunberg tube under vacuum.

‡ Percentage of methylene blue color reduction measured with Lumetron photoelectric colorimeter after the addition of methylene blue solution of 1 cc. of 1:20,000 methylene blue, 1 cc. of 0.05 M dextrose, and 8 cc. of phosphate buffer (pH 7.0).

§ Numerous culture tests (with extinction limit of 10^{-3} mg.) proved absence of viable bacilli.

indicate that the action noted with viable bacilli is present in the bacillary residue from an acetone extracted enzyme preparation from the bacilli.

The findings in table 3 bear out those in table 2 for complete reduction instead of the amount reduced after a definite time interval (four hours) and indicate the significance of the quantity of bacilli in the time of total reduction of the methylene blue. The findings presented in table 3 indicate also that viability is not essential to reduction since the bacillary residue is still active after extraction with acetone at 57°C., as is also a suspension of bacilli heated at 55°C. for thirty minutes (which destroys viability). Likewise, bacilli in watery suspension killed by toluol and xylol at 37°C. still reduce methylene blue, but autoclaving at 118°C. and complete autolysis of the bacilli under toluol for one week at 37°C. destroy this enzymatic action. It is interesting that the re-

TABLE 3

REDUCTION OF METHYLENE BLUE BY HUMAN TUBERCLE BACILLI*

BACILLARY PREPARATION USED	TIME IN HOURS OF 100 PER CENT REDUCTION†				
	Control	1 mg. Streptomycin per cc.	1 mg. Dihydrostreptomycin per cc.	1 mg. PAS per cc.	1 mg. Potassium Cyanide per cc.
Viable bacilli 10 mg. (moist) per cc.—sensitive	2.5	2.5			
5 mg. (moist) per cc.—sensitive	4.0	4.0	4.0	4.0	4.5
Viable bacilli 10 mg. (moist) per cc.—resistant	2.5	2.5			
5 mg. (moist) per cc.—resistant	4.0	4.0	4.0	4.0	4.5
Acetone extracted bacilli dry bacilli extracted 24 hours 57° C. 50 mg. (dry) per cc.‡	3.5	3.5	3.5		
Heat-killed bacilli suspension heated 55° C. 30 minutes 20 mg. (moist) per cc.‡	4.5	4.5			
Nonviable bacilli suspension autoclaved 118° C. 20 mg. (moist) per cc.‡	No reduction	No reduction			
Xylol-treated bacilli suspension 37° C. 4 hours (open) 10 mg. (moist) per cc.‡	4.0				
Toluol-treated bacilli suspension 37° C. 4 hours (open) 10 mg. (moist) per cc.	4.0				
Toluol-killed bacilli suspension 37° C. 15 hours (sealed) 20 mg. (moist) per cc.‡	5.0	5.0			
Autolyzed bacilli suspension autolyzed 7 days 37° C. 50 mg. (moist) per cc.‡	No reduction				

* This tabulation is a composite of triplicate tests performed at 37°C.

† The methylene blue solution used for test consisted of 1 cc. of 1:20,000 methylene blue, 1 cc. of 0.05 M dextrose, and 8 cc. of phosphate buffer (pH 7).

‡ Culture test (sensitive to 10^{-4} mg.) showed absence of viability.

duction of methylene blue under anaerobic conditions by streptomycin-sensitive and streptomycin-resistant human tubercle bacilli is not influenced appreciably by the presence of 1 mg. of streptomycin or dihydrostreptomycin per cc. or by the presence of 1 mg. of para-aminosalicylic acid (PAS) per cc. or 1 mg. of potassium cyanide (KCN) per cc.

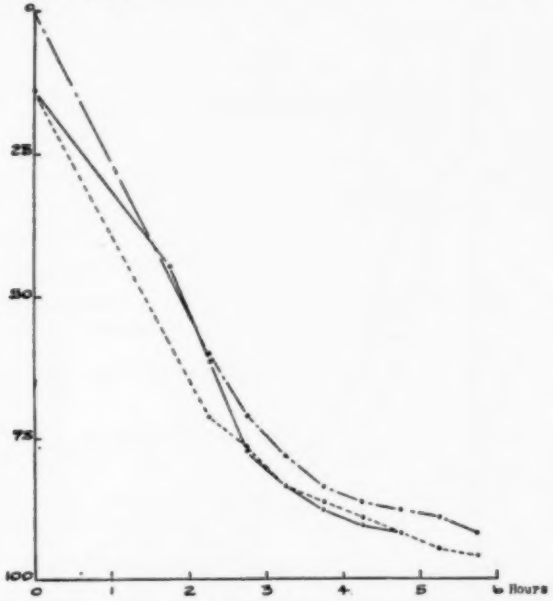

FIG. 1. Effect of streptomycin and dihydrostreptomycin on the reduction of methylene blue by human tubercle bacilli in Locke's solution.

The experiment was performed at pH 7.0.

The ordinate represents the percentage of transmission on the Lumetron.

—— 5 mg. of human tubercle bacilli per cc. of Locke' solution.

—·— 5 mg. of human tubercle bacilli plus 1 mg. of streptomycin per cc. of Locke's solution.

----- 5 mg. of human tubercle bacilli plus 1 mg. of dihydrostreptomycin per cc. of Locke's solution.

Methylene blue = 1:200,000

These findings with added streptomycin and dihydrostreptomycin with sensitive and resistant tubercle bacilli, as determined by methylene blue reduction, are illustrated graphically in figure 1 for streptomycin-sensitive, and in figure 2 for streptomycin-resistant, bacilli.

The Reduction of Sodium Tellurite

In 1905 Gosio described the tellurite and selenite reduction test as an index of bacterial viability, and this reaction was used with the tubercle bacillus by Corper (16) in

1915. Little is known even today, however, regarding its exact mechanism of action. As Sula reported in 1947 (17), "fresh colonies of tubercle bacilli reduce potassium tellurite completely; old colonies show areas without tellurite reduction especially in the center of the colonies. A positive tellurite test indicates apparently the presence of living tubercle bacilli." Thus, the nature of the tellurite reaction of tubercle bacilli appeared still pertinent as a correlation to the findings on enzymes or directly as a factor of dehydrogenases.

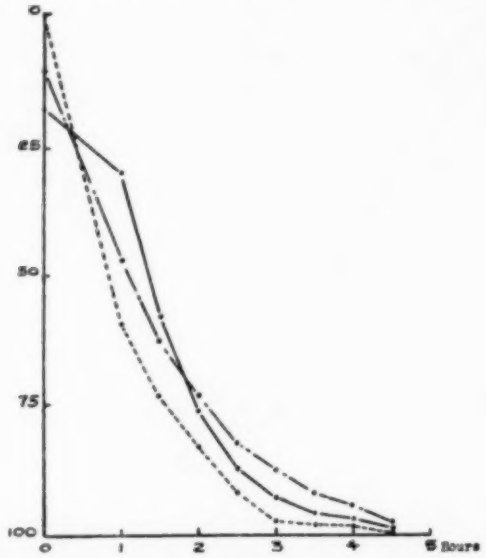

FIG. 2. Effect of streptomycin and dihydrostreptomycin on the reduction of methylene blue by streptomycin-resistant human tubercle bacilli in Locke's solution.

The experiment was performed at pH 7.0.
The ordinate represents the percentage of transmission on the Lumetron.
—— 5 mg. of resistant human tubercle bacilli per cc. of Locke's solution.
– – 5 mg. resistant human tubercle bacilli plus 1 mg. of streptomycin per cc. of Locke's solution.
----- 5 mg. of resistant human tubercle bacilli plus 1 mg. of dihydrostreptomycin per cc. of Locke's solution.
Methylene blue = 1:200,000

As preliminary tests with bacillary colonies, it was found that the reduction of the tellurite salt to a black color, differing from the methylene blue reduction, occurred directly on the bacilli and remained there, possibly because of the insolubility of the reduced product as opposed to that with methylene blue. The optimum pH for the tellurite reaction was approximately pH 7.0 and pH 8.0, and it occurred with viable bacilli equally well in the presence of air or in a vacuum.

Findings in more exacting tests with streptomycin-sensitive and streptomycin-resistant human tubercle bacilli are recorded in table 4.

It is evident from table 4 that, although viable human tubercle bacilli reduce sodium tellurite, such reduction can occur also with nonviable bacilli resulting from acetone extraction for twenty hours at 57°C. and following the use of toluol water-killed bacilli. This reduction can occur both in a nutrient medium or in

TABLE 4

REDUCTION OF SODIUM TELLURITE BY STREPTOMYCIN-SENSITIVE AND STREPTOMYCIN-RESISTANT TUBERCLE BACILLI*

BACILLI AND TREATMENT	REDUCTION TIME IN HOURS Na_2TeO_3 1 MG. PER ML.	
	Nutrient Wong-Weinzirl medium	Non-nutrient phosphate buffer
Sensitive bacilli clumps†	2	1½
Clumps + 1 mg. streptomycin per ml.	1½	1½
Resistant bacilli clumps	2	1½
Clumps + 1 mg. streptomycin per ml.	2	1½
Sensitive bacilli—24 hours contact with 1 mg. streptomycin per ml.	2½	2½
Resistant bacilli—24 hours contact with 1 mg. streptomycin per ml.	2½	2½
Bacilli after 18 hours' contact with toluol water at 37°C. in sealed tube‡	3–5	3–5
Acetone extracted bacilli 20 hours (57°C.) clumps	3–4	3–4
Clumps + 1 mg. streptomycin per ml.‡	3–4	3–4
Heat-killed bacilli 118°C. 30 minutes‡	No reduction	No reduction

* This tabulation is a composite of triplicate tests performed at 37° C. in a phosphate buffer solution at pH 7.0.

† Fine clumps in suspension containing approximately 10 to 20 mg. of moist bacilli per ml.

‡ Culture tests (sensitive to 10^{-8} mg.) showed absence of viability.

a non-nutrient phosphate buffer solution. It is not influenced appreciably by the presence of streptomycin (1 mg. per ml.) nor does the reaction differ for streptomycin-sensitive or streptomycin-resistant tubercle bacilli. Therefore, sodium tellurite reduction does not appear to be a test for viability of tubercle bacilli alone, and the action is apparently of enzymatic nature which can occur in the absence of intact viable cells. The action occurs in a simple phosphate buffer solution in the absence of a nutrient substrate and apparently in intimate relation to the bacillary bodies.

TABLE 5

THE EFFECT OF AGE OF CULTURE AND VARIOUS TREATMENTS OF HUMAN TUBERCLE BACILLI ON THE REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE*

TYPE OF CULTURE MEDIA AND TREATMENT OF THE BACILLI†	10 DAYS	1 MONTH	1.5 MONTHS	2 MONTHS	8 MONTHS	1.5 YEARS AND OVER
Wong-Weinzirl liquid media culture	5‡	5–7	10–12	10–12	15–18	No reduction§
Wong-culture heat-killed bacilli—30 minutes 55°C.‖		25–30				
Wong-culture toluol-killed bacilli—37°C. (sealed) 18 hours.‖		25–30				
Wong-culture heat-killed bacilli—5 minutes 95°C.‖			No reduction			
Wong-culture autolyzed bacilli (toluol water) 7 days 37°C.‖			No reduction			
Wong-culture acetone extracted bacilli—20 hours 57°C. (dry)‖				17–20		
Wong-culture dry bacilli 24 hours vacuo over P_2O_5				17–20		
Whole egg-glycerol broth solid media culture	5	17–20	Partial reduction 2.5 hours	No reduction§		

* This tabulation is a composite of triplicate tests performed at 37°C. in a neutral solution (pH 7.0)

† Reactions tested in 0.05 per cent triphenyltetrazolium chloride solutions. All ten-day cultures on all solid media reduced the triphenyltetrazolium chloride solution in three to five minutes. After one month, the reduction time was usually fifteen to thirty-five minutes, but after two months, most media showed spotty or no reduction.

‡ Time of reduction in minutes of triphenyltetrazolium chloride (0.05 per cent in water).

§ The bacilli are viable since cultures were positive on yolk medium in dilution of 10^{-6} mg. per ml. of plant.

‖ Culture test (sensitive to 10^{-6} mg.) showed absence of viability.

2,3,5 Triphenyltetrazolium Chloride Reduction

The use of tetrazolium salts as a possible indicator for viability of tissues is rather recent and, since the cytoplasm and nuclei of living cells do not stain with any of the vital dyes, tetrazolium (18) was tested as an indicator of cellular viability. As a result of tissue culture studies with chick heart fibroblasts, Stein and Gerarde (19) conclude that the tetrazolium chloride is not an indicator of cell viability, and that "the action of tetrazolium chloride depends on combination with dehdyrogenases which cause a reduction of the dye to its colored form." Apparently the failure of the compound to penetrate through the living cellular membranes is responsible for the negative results obtained. On the other hand, Zwiefach, Black, and Shorr (20) consider tetrazolium salts a new implement in the study of metabolic activity of living cells. The compound is colorless and water soluble and yields a deep red, water-insoluble formazan on reduction. The intracellular reduction of this compound results from its interaction as a hydrogen acceptor with tissue dehydrogenases, the insoluble formazan being deposited locally in the cells in patterns which vary in different tissues.

TABLE 6

THE EFFECT OF STREPTOMYCIN ON THE REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY HUMAN TUBERCLE BACILLI

TYPE OF BACILLI	TIME OF CONTACT WITH 1 MG. OF STREPTOMYCIN PER ML. AND TIME IN MINUTES OF REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE					
	0	1 hour	3 hours	20 hours	3 days	5 days
Streptomycin-sensitive	10–15	10–15	10–15	10–15	10–15	10–15
Streptomycin-resistant	10–15	10–15	10–15	10–15	10–15	10–15

The reaction of tubercle bacilli to the 2,3,5 triphenyltetrazolium chloride (TTC) under varying conditions in definite concentration was studied in the experiment recorded in table 5.

It can be noted from table 5 that, in a 0.05 per cent triphenlyltetrazolium solution in a buffer or nutrient solution, the formazan insoluble red deposit by the bacilli develops visibly within five minutes in a young culture (ten days to one month old) on Wong-Weinzirl synthetic nonprotein medium. As the culture ages, however, the time of reduction to the formazan at 37°C. is fifteen to eighteen minutes at eight months. No appreciable reduction occurs when the bacilli have been incubated at 37°C. for more than one and one-half years although these cultures, as previously noted, contain viable bacilli. On glycerol agar and other solid media, the reduction capacity disappears earlier, being absent in some cases after two months at 37°C. This reduction of TTC by tubercle bacilli appears to be a function of enzymatic reduction action. It is similar to the reduction of sodium tellurite since low-heat destruction (thirty minutes at 55°C.) of the bacilli, acetone extraction, and a short period of exposure to toluol water do not markedly interfere with the reaction, while five minutes at 95°C. completely abolishes the effect.

To determine if streptomycin exerted an effect upon the TTC reaction, it was

tested both in the absence and presence of streptomycin (1 mg. per ml.) on sensitive and resistant human tubercle bacilli. The results are recorded in table 6.

It is evident from table 6 that streptomycin in amount of 1 mg. per ml. does not exert any appreciable effect upon the time of reduction of TTC to the colored formazan regardless of whether it is added to streptomycin-sensitive or streptomycin-resistant human tubercle bacilli.

SUMMARY

It is apparent that enzymatic reactions are a vital part of tubercle bacilli and that qualitatively the tubercle bacilli possess many of the enzymes found in the higher forms of life. It is problematic whether certain chemotherapeutic and antibiotic effects upon the mammalian tubercle bacilli are the results of actions on enzyme systems. In order to define some of these actions, various so-called vital processes and enzymatic processes of the tubercle bacillus have been studied.

In studies on diamine-oxidase (histaminase) in tubercle bacilli, it was found that the human tubercle bacillus is incapable of destroying histamine and, therefore, this mechanism of reaction does not appear to be at the basis of streptomycin action on these bacilli.

An analysis of the reduction of methylene blue (dehydrogenase) by mammalian tubercle bacilli disclosed that the reaction is enzymatic. It does not differ quantitatively for streptomycin-sensitive or streptomycin-resistant human tubercle bacilli with or without the presence of streptomycin.

An analysis of the reduction of sodium tellurite by mammalian tubercle bacilli disclosed that the reaction is not dependent upon the viability of the bacilli, although it is probably enzymatic in nature and does not require the presence of a nutrient substrate fluid. Streptomycin is without appreciable effect on this enzymatic system.

An analysis of the reduction of 2,3,5 triphenyltetrazolium chloride to the colored formazan deposit by mammalian tubercle bacilli is of possible enzymatic nature. To some extent it is influenced quantitatively by the age and type of the culture, young cultures vigorously forming the formazan while old cultures, although definitely still viable, fail to do so in reasonable time. It would appear from these studies that the formazan formation could be used efficiently to determine the state of vitality of the bacillary culture. Streptomycin does not appreciably affect the formazan formation by human tubercle bacilli whether sensitive or resistant to streptomycin. Accordingly, the enzyme involved in the formation of formazan does not appear to be concerned with streptomycin action on tubercle bacilli.

SUMARIO

Las Reacciones Encimáticas del Mycobacterium Tuberculosis y la Acción de la Estreptomicina

Es evidente que las reacciones encimáticas constituyen parte integrante de los bacilos tuberculosos y que, cualitativamente, esos bacilos poseen muchos de los encimas descubiertos en las formas superiores de la vida. Parece problemático si ciertos efectos quimio-

terapéuticos y antibióticos ejercidos sobre los bacilos tuberculosos mamíferos son resultado de las acciones recaídas sobre los sistemas encimáticos. A fin de definir mejor algunas de esas acciones, se han estudiado varios de los llamados procesos vitales y procesos encimáticos del bacilo tuberculoso.

En los estudios de la oxidasa diamínica (histaminasa) en los bacilos tuberculosos, se descubrió que el bacilo tuberculoso humano es incapaz de destruir la histamina y, por lo tanto, ese mecanismo de reacción no parece representar la base del efecto de la estreptomicina sobre dichos bacilos.

Un análisis de la reducción del azul de metileno (dehidrogenasa) por los bacilos tuberculosos mamíferos reveló que la reacción observada es encimática, sin que discrepe cuantitativamente para los bacilos tuberculosos humanos ya sensibles o resistentes a la estreptomicina en presencia o ausencia de esta droga.

El análisis de la reducción del telurito de sodio por los bacilos tuberculosos mamífero reveló que la reacción producida no depende de la viabilidad de los bacilos, sino que es probablemente de naturaleza encimática, y no exige la presencia de un líquido substratal nutriente. La estreptomicina no ejerce efecto apreciable sobre ese sistema encimático.

El análisis de la reducción del cloruro de 2,3,5 trifeniltetrazolio al depósito teñido de formazán por los bacilos tuberculosos mamíferos es posiblemente de naturaleza encimática. Hasta cierto punto la afectan cuantitativamente le edad y el tipo de los cultivos, formando los jóvenes enérgicamente formazán, sin que hagan otro tanto, en plazo razonable, los viejos, aunque todavía decididamente viables.

A juzgar por estos estudios, parece que cabría usar efectivamente la formación de formazán para determinar el estado de viabilidad de los cultivos bacilares. La estreptomicina no afecta apreciablemente dicha formación por los bacilos tuberculosos humanos, ya sean sensibles o resistentes a esa droga. Por consiguiente, el encima que interviene en la formación del formazán no parece figurar en la acción de la estreptomicina sobre los bacilos de Koch.

RESUME

Reactions enzymatiques du Mycobacterium tuberculosis et action de la streptomycine

Il est apparent que les réactions enzymatiques sont une fonction essentielle du bacille tuberculeux et que, qualitativement, le bacille tuberculeux possède bon nombre d'enzymes trouvées dans des organismes d'un ordre plus élevé. Le problème est de savoir si certains effets chimiothérapeutiques et antibiotiques s'exerçant sur le bacille tuberculeux des mammifères sont le résultat d'actions sur le système enzymatique. Dans le but de définir certaines de ces actions, divers processus, soit disant vitaux, ainsi que les processus enzymatiques du bacille tuberculeux, ont été étudiés.

Dans les recherches sur l'oxydase diamine (histaminase) dans le bacille tuberculeux, il été découvert que le bacille tuberculeux humain est incapable de détruire l'histamine et, en conséquence, ce mécanisme de réactions ne paraît pas être à la base de l'action de la streptomycine sur ces bacilles.

Une analyse de la réduction du bleu de méthylène (déhydrogenase) par le bacille tuberculeux humain, a permis de découvrir que cette réaction est d'ordre enzymatique. Quantitativement, il n'y a pas de différence dans la réaction pour le bacille tuberculeux humain streptomycino-sensible ou streptomycino-résistant, qu'il y ait ou non présence de streptomycine.

Une analyse de la réduction du tellurite de sodium par le bacille tuberculeux de mammifère a montré que la réaction ne dépend pas de la viabilité du bacille, qu'elle est probablement de nature enzymatique et ne réclame pas la présence d'un substrat nutritif liquide. La streptomycine n'a aucun effet appréciable sur ce système enzymatique.

Une analyse de la réduction du chlorure de 2,3,5 triphényltétrazolim jusqu'à formation d'un dépot de formazan par le bacille tuberculeux de mammifère indique qu'elle être de nature enzymatique. Jusqu'à un certain point, cette réduction est influencée quantita-

tivement par l'âge et le type de la culture, les cultures jeunes formant vigoureusement le formazan, alors que dans les vieilles cultures il ne ne se produit pas dans un temps raisonnable, même lorsque ces cultures sont nettement encore viables. D'apres ces études, il semble que la formation de formazan pourrait être utilisée avec profit pour déterminer les conditions de vitalité de la culture bacillaire. La streptomycine n'affecte pas de façon appréciable la formation de formazan par le bacille tuberculeux humain, qu'il soit sensible ou résistant à la streptomycine. En conséquence l'enzyme intéressé dans la formation du formazan ne semble pas en relation avec l'action de la streptomycine sur le bacille tuberculeux.

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TUBERCULOSIS AND SICKLE-CELL ANEMIA[1]

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INTRODUCTION

It is frequently stated (1, 2, 3, 4) that patients with sickle-cell anemia commonly die of respiratory infections, especially tuberculosis. This belief seems to be a clinical impression which has not been corroborated by statistical analysis. It is the purpose of the present paper to report a study of the incidence of tuberculosis in post-mortem material by comparing the findings in Negroes with sickle-cell anemia with those in Negroes who had shown no evidence of sickle-cell anemia during life. Note was taken of the presence and types of tuberculosis and of the presence of fibrous pleural adhesions as an index of past inflammation (specific or nonspecific).

OBSERVATIONS

Between 1936 and 1949 there were 27 autopsied cases of sickle-cell anemia at this hospital, all of which occurred in Negroes. A review of the records before 1936 was not feasible. The largest number of cases occurred in persons in the third decade of life, but the age at death varied from six months to sixty-nine years (figure 1A). Likewise, most of the patients in whom the anemia was complicated by tuberculosis were in the third decade at the time of death.

The findings at post-mortem examination in this group were compared to the corresponding findings in a control group of Negro patients. At the Philadelphia General Hospital, almost all of the patients admitted to the tuberculosis division are selected by an agency outside of the hospital, while all patients with sickle-cell anemia are part of the general hospital population. As a consequence, the control group was also selected from the general hospital population.

For a control series, 200 consecutive autopsy protocols in 1936 (excluding patients admitted to the tuberculosis division) and another 200 in 1949 were reviewed.[2] One hundred and thirty Negroes were in this group and they formed the control series. Control cases were sought for both 1936 and 1949 because the mortality rate due to tuberculosis in the general population had dropped approximately 50 per cent from 1936 to 1949 (5). There were no significant differences between the figures for these two years in the control series. The age distribution is shown in figure 1B. In contrast to the sickle-cell anemia series, the majority of patients in the control series were in the fourth to sixth decades at the time of death and the highest incidence of tuberculosis was in the fifth decade.

[1] From the Department of Chronic Diseases of the Chest, Philadelphia General Hospital, and the Woman's Medical College of Pennsylvania, Philadelphia, Pennsylvania.

[2] Patients who died in the first decade of life were excluded from the control series because there was only one such patient in the sickle-cell anemia series.

In table 1 a comparison is made of the findings in the control group and those in the sickle-cell anemia group with respect to the incidence of pleural adhesions and tuberculosis of all types. The results indicate that the incidence of both

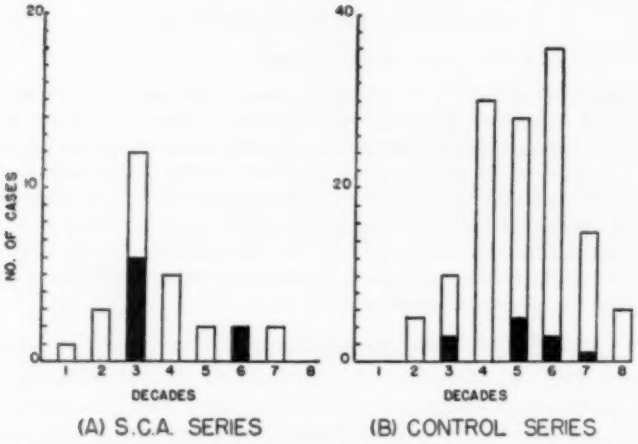

FIG. 1. Age distribution of Negroes in study. Those with tuberculosis are shown in black. (A) sickle-cell anemia (S.C.A.) series, (B) control series.

□ cases without tuberculosis
■ cases with tuberculosis

TABLE 1

INCIDENCE AT POST-MORTEM EXAMINATION OF PLEURAL ADHESIONS AND TUBERCULOSIS IN NEGROES WITH SICKLE-CELL ANEMIA COMPARED WITH NEGROES WITHOUT SICKLE-CELL ANEMIA

	TOTAL NUMBER OF CASES	PLEURAL ADHESIONS		TUBERCULOSIS	
		Number	Per cent	Number	Per cent
Control series 1936	55	24	44	3	5.5
Control series 1949	75	33	44	9	12.0
Total controls	130	57	44	12	9.0
Sickle-cell anemia series 1936–1949	27	22	82	8	30.0

pleural adhesions and of all types of tuberculosis is definitely greater in Negroes with sickle-cell anemia than in Negroes without this disease. These differences are statistically significant.[1] ($P < 0.01$ for pleural adhesions and $P < 0.05$ for tuberculosis.) There may be factors of selection which have not been recognized

[1] By the standard T test for proportions.

and cannot be measured, but it is unlikely that they influence the differences between these groups of patients to such an extent as is evident here.

An attempt was made to compare the incidence of the various types of tuberculosis found at autopsy in these groups of cases, but the breakdown yielded

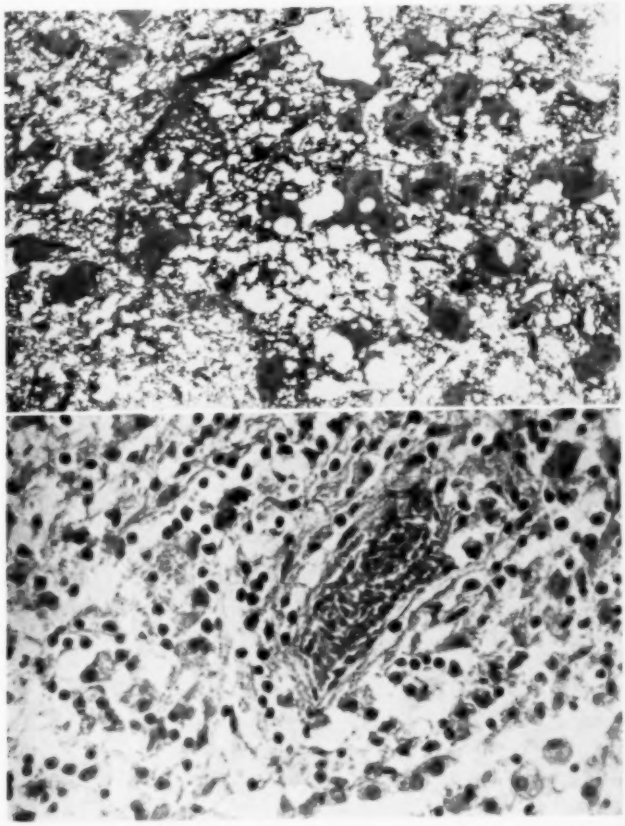

FIG. 2. Photomicrographs of a lung section taken from a patient with sickle-cell anemia and miliary tuberculosis. A. (Upper) low power, to show distribution of tuberculous lesions. B. (Lower) high power photomicrograph of a tuberculous lesion, showing a capillary packed with sickled erythrocytes.

numbers in each subgroup which were too small to allow a statistically valid comparison.

The data presented in figure 1 suggest that tuberculosis occurs at an earlier age in patients with sickle-cell anemia than in those without sickle-cell anemia.

This is presumably due to the fact that Negroes with sickle-cell anemia have tuberculosis more often and do not live as long as do Negroes without this disease. The question arose whether, if patients in the same age group were

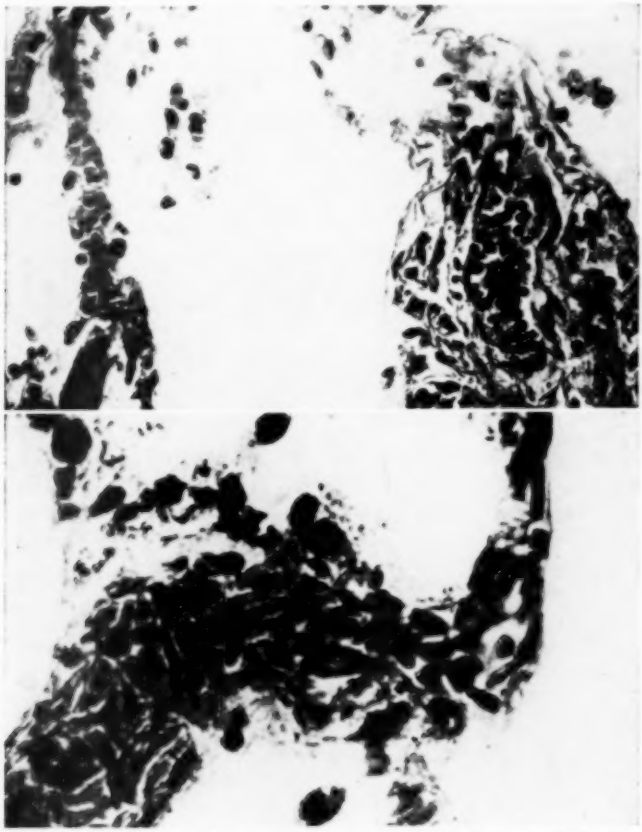

FIG. 3. Photomicrographs of same lung section shown in figure 2. Both pictures are taken from relatively normal lung tissue between the tuberculous lesions. A. (Upper) shows normally shaped erythrocytes in the capillaries. B. (Lower) shows a capillary packed with sickled erythrocytes.

compared, similar findings would appear. Since the largest number of patients with sickle-cell anemia died in the third decade, this age group was scrutinized more closely. Unfortunately, the patients in this age group with sickle-cell anemia numbered only 12, but 6 (50 per cent) had some form of tuberculosis and 9 (75 per cent) showed pleural adhesions. The control series had a total of only

10 patients in the third decade of life. Therefore, this age group was enlarged by reviewing 1,000 consecutive autopsy protocols in the years 1936–1937 and another 1,000 in the years 1949–1950. In this way, 40 autopsy protocols were found of Negro patients who had died in the third decade of life and who had been admitted to departments other than the tuberculosis division. Of these, only 10 (25 per cent) had tuberculosis in some form and only 14 (35 per cent) showed pleural adhesions. Thus, pleural adhesions and tuberculosis were twice as frequent in Negroes with sickle-cell anemia as in Negroes without the anemia. However, the difference was statistically significant only for pleural adhesions ($P < 0.05$) and not for tuberculosis.

The lung sections of one case in the sickle-cell anemia series were very instructive, especially because scattered small tuberculous lesions were separated by relatively normal lung tissue (figure 2A). Within *all* tuberculous lesions (figure 2B), *all* vessels were filled with sickled red cells. This was also true in other cases. In the normal lung tissue, however, some areas showed vessels which contained normally shaped red cells (figure 3A), while other areas showed vessels which contained sickled red cells (figure 3B). A particular effort was made to see whether there were some arteries, especially those in relation to bronchioles, within tuberculous tissue which would show normal erythrocytes while other vessels within the tuberculous tissue showed sickled erythrocytes. No such distinction could be made.

DISCUSSION

It is generally believed that tuberculosis in the Negro differs from tuberculosis in the white race (6, 7, 8, 9, 25). The case mortality is greater and the course of the disease is more acute in the Negro. Nevertheless, the cause of this difference is controversial. Some writers believe that the cause is a socio-economic difference, while others believe it is due to differences in racial susceptibility to tuberculous infection. It is probable that both views are correct and there is some evidence to indicate that the Negro has less native resistance to the disease than do white persons. This may well be due to natural selection, for, generally speaking, the Negro race has been exposed to tuberculosis for a much shorter time than has the white race.

Since tuberculous lesions may be affected by oxygen supply, and hence by vascular phenomena, it is conceivable that the racial characteristics of sickle-cell anemia and the sickling trait may be factors in the lack of resistance to tuberculosis manifested by some Negroes. A later paper will deal with the relationship between the sickling *trait* and tuberculosis; a relationship which is of great importance because the sickling *trait* is relatively common in the Negro. The present paper deals with the relationship between sickle-cell *anemia* and tuberculosis.

Standard textbooks on tuberculosis do not mention sickle-cell anemia (7, 10, 11, 12). The literature is almost nonexistent on the combination of the 2 diseases. Hamlin (13) reported a single case. Cabral (14) discusses 2 patients with sickle-cell anemia who had acute pulmonary tuberculosis. Therapy for tuber-

culosis in these 2 cases was unsuccessful. On the basis of such limited data, Cabral concluded that sickle-cell anemia predisposes the patient to tuberculosis and that sickle-cell anemia is an important factor which explains why tuberculosis tends to be more acute in the Negro than in the white race.

The periodical literature on sickle-cell anemia and textbooks of hematology suggest that pulmonary infection, particularly tuberculosis, is common in patients with sickle-cell anemia. The present investigation tends to corroborate this impression.

The question now arises why tuberculosis should be more common in Negroes with sickle-cell anemia than in Negroes without sickle-cell anemia. Thought is naturally directed to the roles that sickled erythrocytes and the vascular sequelae of sickle-cell anemia may play in the inflammatory process. One must reconcile the fact that the tubercle bacillus is a strict aerobe (7, 15) with the fact that a common lesion in sickle-cell anemia is a vascular obstruction by sickled cells accompanied by anemia, resulting in tissue anoxia. If, as suggested, sickling is a factor in tuberculous disease, it may affect either tissue resistance or the tubercle bacillus itself. Let us consider the possibilities.

It has been observed in the present study that, under suitable conditions, the blood in a case of sickle-cell anemia flowing through either relatively normal lung tissue or through a tuberculous pulmonary lesion may sickle; whereas, simultaneously, it may remain unsickled in some areas of normal lung tissue (figure 3). The presence of both sickled and unsickled cells in the same section of lung probably answers the objection that sickling seen in post-mortem lung sections is merely an agonal event and does not occur during life.

A factor which has been shown to aggravate tuberculosis is obstruction of the pulmonary arterial circulation. There are two separate circulations through a tuberculous lesion in the lung, one of which (pulmonary arterial) may be obstructed by sickled erythrocytes (as a result of the lower oxygen tension and pH in this system). Since bronchial arterial blood is oxygenated systemic blood, it may be that this system does not contain sickled erythrocytes. Therefore, under such conditions, the tuberculous lesion may be supplied by only one circulation (bronchial arterial) which carries well-oxygenated blood. One could assume, then, that the oxygen tension is higher in the tuberculous lesions of patients with sickle-cell anemia than in normal lung tissue or in tuberculous lesions of patients without sickle-cell anemia. Such a situation would aid the tubercle bacillus in its battle against the host. This is a plausible explanation which has been invoked to explain why tuberculosis becomes worse when the pulmonary artery is ligated or when a systemic artery is anastamosed to a pulmonary artery (16, 17, 18).

The importance of the bronchial arterial circulation has been demonstrated, for in chronic lung disease such as tuberculosis the bronchial arteries increase greatly in size (19, 20, 21). This explanation, however, is probably *not* valid in patients with sickle-cell anemia because, in spite of careful search of the lung sections in sickle-cell anemia patients with pulmonary tuberculosis, the present writers have been unable to demonstrate that some vessels within tuberculous

lesions contain normally shaped erythrocytes, as one would expect in the bronchial arterial circulation. *All* vessels *within* tuberculous lesions in the cases studied were packed with sickled erythrocytes.

Another explanation is possible. The tubercle bacillus is a strict aerobe and, within certain limits, it grows better in atmospheres of higher oxygen tension (22). The present discussion is confined to those bacilli spreading into uninvolved alveoli. In the lungs of patients with sickle-cell anemia, conditions in the pulmonary arterial blood are most conducive to sickling because the oxygen tension is low (35 mm. of mercury) and the carbon dioxide tension is high (47 mm. of mercury) (23). Sickling occurs at an oxygen tension below 45 mm. of mercury (24). The writers are unable to say why sickling occurs in some alveolar capillaries and not in others in normal lung tissue but such seems to be true, as illustrated in figure 3. Now, let us assume that sickling obstructs an alveolar capillary. If this obstruction lasts for a significant period of time, then oxygen uptake by the capillary blood from the alveolar air must cease and the affected alveolus should contain a higher concentration of oxygen than does an alveolus from which oxygen continues to be removed by freely flowing blood in the alveolar wall. Let us assume that tubercle bacilli are aspirated into an alveolus whose capillary is obstructed. Growth of the bacilli may be better in such an alveolus than in one whose capillary contains freely flowing blood. Hypothetically, such a condition may enhance a tuberculous infection and cause it to assume clinical proportions more often in patients with sickle-cell anemia than in those without sickle-cell anemia. This hypothesis, of course, only explains what may happen at the periphery of a tuberculous lesion or in freshly infected alveoli. It does not refer to what happens in the depths of a tuberculous lesion where alveolar air no longer penetrates. In such inflammatory tissues, local acidosis and anoxia should enhance sickling. The obstructed blood supply may thus strangle the resistance of the infected tissue by decreasing the supply of defense factors and nutrients.

The present writers do not know whether any of the suggested explanations are correct. Nevertheless, irrespective of the mechanism involved, the findings herein presented support the idea that sickle-cell anemia is a definite factor in the response of some Negroes to tuberculous infection.

CONCLUSIONS

A pathologic study was made of tuberculosis among Negroes with and without sickle-cell anemia. Negroes with sickle-cell anemia show a higher incidence of pleuropulmonary inflammatory disease, as evidenced by a higher incidence of pleural adhesions and a higher incidence of the various forms of tuberculosis, than do Negroes without sickle-cell anemia. The possible explanations of this phenomenon are discussed.

CONCLUSIONES

La Tuberculosis y la Drepanocitemia

Este estudio patológico versó sobre la tuberculosis en los negros con y sin anemia por células falciformes. Los afectados por dicha anemia, comparados con los indemnes, mues-

tran una proporción mayor de afección inflamatoria pleuropulmonar, según evidencian la mayor incidencia en ellos de adherencias pleurales y de las varias formas de tuberculosis. Discútense las posibles explicaciones de ese fenómeno.

CONCLUSIONS

La tuberculose et l'anémie à hématies falciformes

Il a été procédé à une étude pathologique de la tuberculose chez des nègres atteints, ou indemnes, d'anémie à hématies falciformes. Chez les nègres atteints d'anémie à hématies falciformes la fréquence de manifestations inflammatoires pleuropulmonaires était plus élevée, comme le montraient la fréquence plus élevée d'adhérences pleurales et des diverses formes de la tuberculose, que chez les négres indemnes d'anémie à hématies falciformes. Diverses explications possibles de ce phénomène sont discutées.

Acknowledgment

We are grateful to Paul Meier, Ph.D., statistician of the Philadelphia Health and Tuberculosis Association; and to the Department of Laboratories of the Philadelphia General Hospital for permission to use the autopsy material.

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Case Reports

SPONTANEOUS HEMOPNEUMOTHORAX[1,2]

A Review with a Report of Two Cases

DONALD A. FUSIA, JR., AND W. LEIGH COOK, JR.

(Received for publication December 10, 1951)

When spontaneous pneumothorax is associated with intrathoracic bleeding of minimal extent, conservative observation is indicated. If, however, the hemothorax progresses to any considerable extent the blood must be entirely removed, and at an early stage, if complete recovery is to be expected. The following two cases illustrate instances where specific therapy was indicated, one radical and one of a more conservative nature.

CASE REPORTS

Case 1 (E. A.). This 23-year-old insurance salesman was first seen at the Pittsburgh Eye and Ear Hospital on January 11, 1951 for the correction of prognathism. Under local and pentothal anesthesia, mandibular pins were inserted as the first stage of a bilateral resection of the mandible.

On admission to the hospital, the evening of January 20, 1951, he was orthopneic and slightly cyanotic. The pulse was 90 per minute; blood pressure, 90 systolic, 20 diastolic in mm. of mercury. Expansion of the left chest was decreased and breath sounds and fremitus were absent over the lower two-thirds of the left lung. The percussion note was unimpaired. The heart sounds were distant with a suggestion of an apical friction rub. With the exception of the fact that the mouth could not be opened because of jaw splints, the remainder of the examination was not remarkable.

The blood count on admission was: erythrocytes, 4,500,000 per cu. mm.; hemoglobin, 87 per cent; leukocytes, 11,600 per cu. mm. with polymorphonuclears, 66 per cent; lymphocytes, 28 per cent; monocytes, 1 per cent; eosinophils, 4 per cent. The patient was placed in an oxygen tent and sedated. The next day there were signs of fluid in the left chest, and the heart was shifted to the right. The blood pressure had improved to 110 systolic, 70 diastolic. A chest roentgenogram taken January 22, 1951 (figure 1), showed a hydropneumothorax with considerable collapse and adhesions. The chest was half filled with fluid, and the heart was slightly shifted to the right. A diagnostic thoracentesis produced 100 cc. of "pure blood."

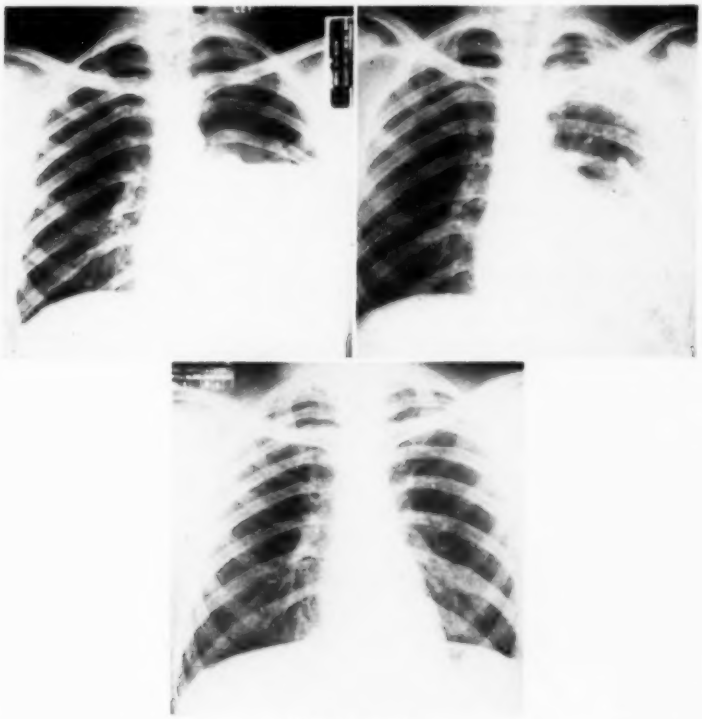
On the third hospital day the erythrocyte count had fallen to 3,000,000 per cu. mm. with 64 per cent hemoglobin. Dyspnea was marked and 1,100 cc. of blood were removed from the left chest. The temperature ranged from 100° to 102° F., and the patient was given 300,000 units of penicillin every twelve hours.

On the fifth day, January 26, 1951, a chest film showed an increase in fluid. The erythrocyte count was 2,900,000 per cu. mm. with hemoglobin of 64 per cent. Dyspnea was less, but pallor was marked. The patient was seen in consultation by a chest surgeon,

[1] From the Medical Service, Veterans Administration Hospital, Aspinwall, Pennsylvania.

[2] Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

and under-water catheter drainage was started. Much air and blood were removed initially and there was bubbling in the jar, indicating a bronchopleural fistula. He was given a whole blood transfusion of 500 cc. Repeated fluoroscopy and chest roentgenograms showed the lung to be re-expanding (figure 2). Recovery was uneventful. At discharge the erythrocyte count was 4,200,000 per cu. mm. with hemoglobin 81 per cent; the leukocyte count was 11,103 per cu. mm.



Case 1 (E. A.)

FIG. 1. (Left) Roentgenogram dated January 22, 1951, showing left hydropneumothorax.

FIG. 2. (Right) Roentgenogram dated January 28, 1951, showing chest catheter in place; lung re-expanded.

FIG. 3. (Bottom) Roentgenogram dated March 11, 1951, showing left lung completely re-expanded.

There was no history of previous significant illness, cough, night sweats, fever, or known exposure to tuberculosis. Several sputum examinations were negative for acid-fast bacilli.

The patient remained asymptomatic but a follow-up chest film obtained on February 21, 1951 demonstrated a small pneumothorax on the right. Subsequent films (figure 3) have shown both lungs fully re-expanded, and no parenchymal lesion has been demonstrated.

Case 2 (C. E. H.). A 25-year-old white, steel mill carpenter was admitted to the Aspinwall Veterans Administration Hospital on August 7, 1950 with the complaint of chest pain and gradually increasing dyspnea of nine days' duration. The past medical history was not significant.

On July 30, 1950 the patient had noted the sudden onset of sharp, stabbing, persistent, anterior chest pain aggravated by inspiration. The pain gradually subsided although medication was required for several days to induce sleep. Four days after the onset of the chest pain he noted increasing dyspnea, at first with effort but eventually persisting at rest. He developed a mild, dry cough aggravated by recumbency. A roentgenogram taken by his local physician revealed the presence of air and fluid in the left chest. Following this, a thoracentesis produced about 500 cc. of "dark red blood." During the previous month the patient had lost approximately 18 pounds.

Examination on admission revealed a thin, well-developed, young white male who was ambulatory but appeared to be in mild respiratory distress. The lips were slightly cyanotic. A respiratory lag was noted on the left, with flattening of the intercostal spaces. The entire left lower chest was dull to percussion and breath sounds were absent over the same area. Egophony and pectoriloquy were noted above the mid-scapular area on the left. Abdominal respiration was marked. Blood pressure was 118 systolic, 70 diastolic in mm. of mercury; pulse was 96 per minute.

The chest roentgenogram obtained on admission to the hospital (figure 4) disclosed a left hydrothorax extending to the level of the fourth rib posteriorly, with a pneumothorax above this level. The mediastinum was shifted considerably to the right. The admission blood count was: erythrocytes, 3.2 million per cu. mm.; hemoglobin, 9 gm. per 100 cc.; leukocytes, 1,500 per cu. mm. with 85 per cent neutrophils, 13 per cent lymphocytes, 1 per cent monocytes, and 1 per cent eosinophils. Prothrombin time was 16 seconds; control, 15 seconds. A serologic test for syphilis was negative; urinalysis was normal. The erythrocyte sedimentation rate was 35 mm. per hour. The cutaneous reaction to tuberculoprotein (PPD, strengths 1 and 2) was negative. Bleeding time was one minute; coagulation time was four minutes.

The patient had a low-grade fever with daily elevations to 100° and 101° F. The pulse and blood pressure remained within normal limits. On the third hospital day, 600 cc. of bloody fluid were removed from the left chest by thoracentesis. A roentgenogram of the chest revealed no appreciable change (figure 5). On the seventh day, 1,500 cc. of bloody fluid were removed, following which roentgenographic examination revealed a decrease in the hydropneumothorax with a shift of the mediastinum back toward the left. He received two whole blood transfusions of 500 cc. The erythocyte count became apparently stable at 3,400,000 cells per cu. mm. with 10.5 mg. of hemoglobin per 100 cc.

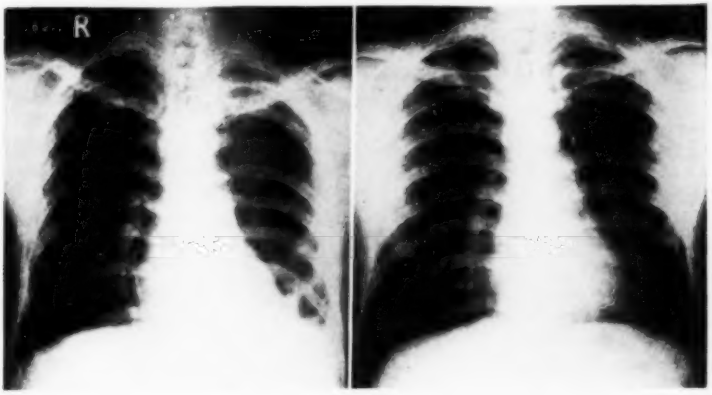
Smear and culture of the pleural fluid were negative for tubercle bacilli and neoplastic cells. The centrifuged sediment consisted of erythrocytes and a few lymphocytes. A guinea pig inoculation was later reported as negative.

Efforts were made to dry the pleural cavity by repeated aspiration but two successive taps were unproductive although the presence of fluid and air were still demonstrable on chest roentgenograms. It was inferred that an organizing blood clot was present. On August 21, 1950 a left thoracotomy was performed under endotracheal anesthesia. The pleural cavity was filled with a large amount of old blood and adherent clot which had collapsed both lobes almost completely. There were many adhesions between the lower lobe and the diaphragm. A half dozen small cystic structures were noted in the apex of the left upper lobe, one of which had ruptured and apparently accounted for the hemopneumothorax. The cystic area was resected and the lung decorticated, which allowed both lobes to reexpand. All remaining clots and blood were removed. One million units of penicillin and 1 gm. of streptomycin were placed in the pleural cavity and the chest was closed with a No. 30 drainage catheter in the tenth intercostal space. During the operation and immediately postoperatively, the patient received an additional 1,000 cc. of whole blood. For a short period, serosanguinous fluid drained from the catheter. Three hundred thousand units of

Case 2 (C. E. H.)

FIG. 4. (Left) Roentgenogram showing left hydropneumothorax.

FIG. 5. (Right) Roentgenogram showing continued left hemopneumothorax after many aspirations.



Case 2

FIG. 6. (Left) Roentgenogram dated September 12, 1950, approximate date of discharge from the hospital.

FIG. 7. (Right) Roentgenogram dated March 15, 1951 (follow-up film).

penicillin were given intramuscularly every twelve hours. Recovery was uneventful. At discharge on the thirty-eighth hospital day, roentgenographic examination showed that the lung was almost completely re-expanded (figure 6).

When re-examined three and six months later (figure 7) the patient had regained his weight.

Resected specimen: The resected specimen consisted of a blood clot from the pleural cavity and a portion of lung tissue. The former consisted of two pieces, one of which was a membranous tag measuring 10 by 7 cm. and up to 1 cm. in thickness. One surface was

covered by a smooth yellowish-red membrane containing flecks of anthracotic pigment. The opposite surface revealed a stringy laminated blood clot which varied from pale tan to dark red in color. The other fragment consisted entirely of spongy blood clot, most of which was pale tan and measured 8.5 by 3 by 1.5 cm. in its greatest dimensions. The portion of lung measured 2.8 by 2 by 0.4 cm. and was covered on one surface by a relatively smooth membrane containing tags of fibrous tissue. The cut surfaces were dark brown with deposits of anthracosis and thickened fibrous septa.

Several portions of the blood clot were covered by a layer of vascular fibrous tissue which was adherent to the pleura in some areas. The section of lung was covered by thin pleura containing some anthracotic pigment. Beneath it the parenchyma was largely replaced by dense scar tissue. Remnants of alveoli remained as small spaces lined by prominent cuboidal cells and filled by pigment-laden macrophages. Beneath this, the relatively intact alveoli were collapsed.

The diagnoses were (*1*) organizing hemothorax and (*2*) chronic fibrous subpleural pneumonitis.

DISCUSSION

Idiopathic spontaneous hemopneumothorax associated with intrapleural hemorrhage in an otherwise healthy person has been but infrequently reported. Its true incidence is difficult to determine. Isolated cases are recorded in reports dealing with spontaneous pneumothorax and with thoracic surgical procedures or under misleading titles such as hemorrhagic pleurisy. The strong possibility that many chests strapped for acute pleurisy and many chest films showing an obliterated costophrenic angle actually represent this entity or its residuum must remain a matter of conjecture. Hartzell (1) in 1942 was able to collect 43 cases. Since that time articles dealing with various aspects of the subject have appeared with increasing frequency until at present it was possible to find reports of some 80 cases.

As with simple spontaneous pneumothorax, the majority of patients fall into the 20- to 40-year age group. Similarly, there is a striking predilection for the male, only two cases having been identified in females (1, 2).

Helwig and Schmidt (3) in 1945 collected the findings in fourteen autopsied cases. In these, most patients had apical pleural scars and six had adhesions. Emphysematous bullae were present in ten, ruptured bullae in four; there were torn pleural adhesions in five, and microscopic examination of two of these revealed them to be highly vascular. In half the victims no bleeding site could be demonstrated. Arst, Lahey, and Kunkel (4) reported an autopsied case showing apical scarring and fibrosis. One torn adhesion was unequivocally demonstrated to be the site of bleeding. In the case in the present report in which thoracotomy was performed, a ruptured bulla was found which was regarded by the surgeon as the source of both the pneumothorax and the hemorrhage.

Of the several explanations offered for the development of spontaneous pneumothorax, the rupture of a valve vesicle on or near the pleural surface seems most plausible (1). A congenital anomaly or pulmonary infection may give rise to a scar so situated that a one-way valve is present at the bronchiolar or alveolar entrance. A bleb forms which gradually enlarges and may rupture during exertion or even in the course of normal respiratory movements. If, as has been shown by Mazzei and Pardal (28), these bullae should be highly vascular or if adhesions are present and torn, a hemopneumothorax may result. The blood supply of the parietal pleura is derived from the systemic circulation; that of the visceral pleura is from the pulmonary and ramifications of the bronchial arteries (22). In a particular case it is almost impossible to determine whether bleeding is systemic or pulmonary in origin. As the systemic arterial pressure is much

greater than the pulmonary pressure, however, a torn adhesion bleeding peripherally would best explain massive hemorrhage. Thus, it is pointless to attempt to control the hemorrhage by compression of the lung with artificial maintenance of the collapse.

Although active tuberculosis seldom develops in patients with pneumothorax or hemopneumothorax who have been followed for significant periods, this possibility cannot be overlooked. Beatty (6) reported a case of a young Negro male who developed cavitary tuberculosis in the homolateral lung four months after an episode of spontaneous hemopneumothorax. Even though this lung had been normal at the time of the accident, however, the contralateral lung had contained minimal active disease. Birch (7) observed a fatal case in which autopsy disclosed a minute apical tuberculous cavity. A case was excluded from the present series because one pleural fluid culture revealed *M. tuberculosis*. Bronchoscopy in this case revealed an elevated hyperemic area interpreted histologically as chronic bronchitis. Although a two-year period of follow-up observations had shown no parenchymal lesion, the etiology was still considered to be tuberculous. The view was once held that tuberculosis was the cause of almost all spontaneous pneumothoraces. The still oft-quoted work of Birch (7) in 1880 lent strength to this opinion. He demonstrated tuberculosis as the cause of 715 of 918 cases of spontaneous pneumothorax. It is to be noted that these were all cases studied post mortem, and idiopathic spontaneous pneumothorax seldom terminates fatally.

Although pneumothorax is a common complication of tuberculosis, intrapleural hemorrhage is not often seen (19). Serous or purulent effusions are frequent. As pointed out by Hyde and Hyde (9), tuberculous spontaneous pneumothorax is usually secondary to subpleural caseation with erosion and rupture of the visceral pleura. If one considers that tuberculous tissue is poor in blood supply, this apparent discrepancy is at least partially explained. In any event, the underlying disease is almost always obvious. Bleeding may occasionally occur from adhesions torn during therapeutic pneumothorax.

The clinical picture of hemopneumothorax may present itself in various degrees of severity. The onset may occur at rest or with activity. Occasionally it follows a sudden increase in intrapulmonary pressure such as coughing or sneezing. A constant early symptom is pain. It is usually sudden, sharp, continuous, and aggravated by movement. It may remain localized to the side of the chest involved or radiate to the shoulder, abdomen, or opposite side. In several reported instances the pain was confined to the abdomen. In one of the cases in the present report, laparotomy was performed before the true nature of the illness was discovered. If the bronchi are sufficiently distorted by the collapse, a dry cough may develop. Not infrequently the initial pain persists for hours or days. This latent interval is followed by a recurrence of pain and signs of intrapleural fluid. This sequence of events may be explained as follows: The initial pain is due to the rupture of a bleb and collapse of the lung. As the lung gradually collapses further, the adhesions are stretched and eventually rupture with resultant hemorrhage. In contrast to simple pneumothorax, a low-grade fever and slight leukocytosis are frequently present. Dyspnea of some degree is present in the majority, and cyanosis is seen in a few of the persons with hemopneumothorax.

The physical signs are those of pneumothorax combined with signs of pleural effusion. Textbook articles usually describe the chest in pneumothorax as being hyper-resonant. As mentioned in the introduction, small or even moderately large spontaneous pneumothoraces will go unsuspected if one relies on this sign. Hyper-resonance is not unusual, however, particularly in tension pneumothorax or with a high degree of collapse. On the basis of the present writers' experience in the examination of patients with therapeutic pneumothorax and of several with spontaneous pneumothorax comparable in size and

degree of negative intrathoracic pressures, it is believed that markedly decreased breath sounds and the absence of dullness are a more suggestive combination. When bleeding into the chest is superimposed upon the pneumothorax, the usual signs of fluid may be demonstrated. If the hemorrhage is massive, circulatory collapse and anemia become evident.

The roentgenographic picture is that of hydropneumothorax. After re-expansion of the lung, apical bullae may occasionally be visualized. It has been stated that lateral pleural adhesions are seldom seen in patients with spontaneous pneumothorax. In one of the present cases, adhesions were demonstrated by roentgenographic examination.

The diagnosis of hemopneumothorax should be relatively simple. Pleural fluid of any substantial amount appearing early in the course of spontaneous pneumothorax is almost certainly blood. A serous effusion is distinctly unusual (9, 11, 16). Even in therapeutic pneumothorax, effusion does not usually appear for one to six months (12). In 76 cases of spontaneous pneumothorax studied by Hyde and Hyde (9), fluid was present above the level of the diaphragm in only four and in all of these it was blood. A diagnostic thoracentesis should always be done. An erythrocyte count and hematocrit determination should be made on the aspirate to ascertain that it is blood and not merely bloody fluid. Berliner (13) demonstrated that 5,000 to 6,000 erythrocytes per cu. mm. will give a rosy tint to pleural fluid. Counts higher than this may closely simulate blood. The causes of hemorrhagic effusion are numerous but, with the possible exception of tuberculosis, lung abscess, and (rarely) pulmonary infarction (14), none are ordinarily associated with pneumothorax. Even if adequate visualization of the lung parenchyma by roentgenography is obscured temporarily by effusion, a history of chronic illness can be elicited.

Experience gained in the management of chest wounds during and since World War II has drastically altered the method of treatment in hemopneumothorax (15). Therapy should be directed toward the relief of increased intrapleural pressure, control of shock and hemorrhage, and prevention of impairment of pulmonary function. Previously it was believed that the aspiration of blood should be delayed and, when carried out, replaced with air. As in massive gastrointestinal bleeding, transfusions were resorted to with reluctance for fear of inciting further hemorrhage. Reliance was placed on a lowered blood pressure and increased intrapleural pressure to staunch the hemorrhage. It is now known that the lung should be re-expanded as rapidly as possible by frequent thoracentesis. Air replacement is useless. The vital signs should be closely observed. Shock and anemia should be treated by adequate replacement with whole blood. There is no evidence that properly administered whole blood incites further hemorrhage.

It was once thought that blood in the pleural space did not clot. Christian's most recent textbook of medicine expresses the view that the pleura has an anticoagulant action. According to LeBlanc and Trousseau (18), the blood actually clots but is rapidly defibrinated by the churning action of the heart and respiratory movements. The fibrin is deposited on the pleura, or shreds and small clots float about in the liquid blood. By placing blood withdrawn from the femoral vein into the thoracic cavities of dogs, Melick and Spooner (20) showed that clotting took place normally. Cosgriff (19) studied blood from a case of traumatic hemopneumothorax by means of coagulation time, prothrombin time, recalcified plasma time, erythrocyte sedimentation rate, and fibrinogen levels. No unusual anticoagulant activity could be demonstrated. He concluded that the blood had already clotted normally.

Even though it has been shown that the pleura in many or even most cases will resorb large amounts of blood, fibroblastic proliferation may appear within five days

(20). This leads to organization and formation of a "peel" over the contracted lung surface. If this occurs, pulmonary function may be seriously jeopardized. Thoracotomy will then be necessary to obviate pulmonary dead space and ensure full and prompt re-expansion of the lung. If this "peel" is allowed to remain, the eventuality of chronic fibrothorax or even empyema is imminent.

Read and Berry (21) recently reported the use of streptokinase and streptodornase in the successful treatment of a case of spontaneous hemopneumothroax which could no longer be aspirated. It is to be hoped that wider use of these enzymes will reduce or eliminate the necessity for surgery.

SUMMARY

The pertinent literature relative to spontaneous hemopneumothorax has been reviewed and two illustrative cases of this clinical syndrome, one treated conservatively and one treated by operative intervention, have been reported.

On the basis of this material, the following criteria for managing spontaneous hemopneumothorax are recommended:

When spontaneous pneumothorax is associated with evidence of pleural fluid, a diagnostic chest tap is indicated.

If blood or grossly bloody fluid is found, it should be evacuated as completely and thoroughly as possible.

Patients should be closely observed by means of fluoroscopy and frequent physical examinations to ascertain whether intrathoracic hemorrhage has ceased.

Whole blood transfusions should be given as indicated. There is no evidence that transfusions prolong hemorrhage.

If for any reason adequate aspiration cannot be carried out, surgical intervention may be necessary (a) to evacuate the blood clot, (b) to remove constricting fibrin peel from the lung surface, or (c) to control hemorrhage when necessary.

The recent use of streptokinase and streptodornase may provide a valuable additional technique in the management of hemothorax.

SUMARIO

Hemoneumotórax Espontáneo: Repaso de la Literatura con Presentación de Dos Casos

Después de repasar la literatura relativa al hemoneumotórax espontáneo, descríbense 2 casos típicos: uno tratado conservadoramente y el otro tratado con la intervención cruenta.

A base de esos datos, ofrécense las siguientes normas para la asistencia del hemoneumotórax espontáneo:

Si el neumotórax espontáneo está asociado a signos de líquido pleural, está indicada un punción diagnóstica del tórax.

Si se descubre líquido sanguinolento, hay que evacuarlo lo más completa y perfectamente posible.

Los enfermos deben ser cuidadosamente observados por medio de la roentgenoscopia y de frecuentes exámenes físicos para averiguar si ha cesado la hemorragia intratorácica.

Las transfusiones de sangre íntegra serán administradas si están indicadas, no habiendo pruebas de que prolonguen la hemorragia.

Si por cualquiera razón no puede llevarse a cabo una aspiración adecuada, quizás sea necesaria la intervención cruenta para: (a) evacuar el coágulo sanguíneo, (b) retirar de la cara del pulmón la capa constrictora de fibrina, o (c) cohibir la hemorragia cuando sea menester.

El reciente empleo de la estreptoquinasa y la estreptodornasa puede aportar otra valiosa adquisición a la asistencia del hemotórax.

RESUME

Hemopneumothorax—Revue de la littérature et observation de deux cas

Revue de la littérature pertinente se rapportant à l'hémothorax spontané et présentation des observations de deux cas illustrant ce syndrome clinique; l'un des cas ayant été l'objet d'un traitement conservateur, l'autre d'une intervention chirurgicale.

D'après cel éléments, les critères pour le traitement de l'hémopneumothorax spontané sont les suivants:

Quand le pneumothorax spontané s'accompagne de signes d'épanchement pleural, une ponction diagnostique de la plèvre est indiquée.

La présence d'un liquide sanguinolent, ou de sang, réclame une ponction évacuatrice aussi minutieuse et complète que possible.

Les malades devront être soumis à une surveillance étroite, avec l'aide de la radioscopie et d'examens physiques répétés, dans le but de s'assurer que l'hémorragie a cessé.

Des transfusions de sang total devront être pratiquées, en cas de besoin. La preuve que les transfusions prolongent l'hémorragie n'a pas été obtenue.

Si, pour une raison quelconque, il n'est pas possible de réaliser une ponction adéquate, l'intervention chirurgicale peut s'imposer dans le but de: (a) évacuer les caillots, (b) débarrasser la surface du poumon de la couche de fibrine, (c) de juguler l'hémorragie en cas de besoin.

L'usage de la streptokinase et de la streptodornase, dont l'emploi est récent, pourrait constituer un procédé additionnel de valeur dans le traitement de l'hémothorax.

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Notes

DEMONSTRATION OF INCREASED DRUG RESISTANCE OF TUBERCLE BACILLI FROM PATIENTS TREATED WITH HYDRAZINES OF ISONICOTINIC ACID[1]

In the early clinical studies published to date[2, 3, 4] on the antituberculous activitity of isonicotinic acid hydrazide[5] and its isopropyl derivative, little has been mentioned regarding the emergence of drug-resistant strains of tubercle bacilli in sputum cultures, although many patients with chronic cavitary disease and sputum positive for tubercle bacilli have been under treatment with these drugs for some months. It has been shown[6, 7] that strains of tubercle bacilli with gradually increasing drug resistance can be produced *in vitro* by serial transfers in liquid medium containing isonicotinic acid hydrazide or its isopropyl derivative. In this manner, strains of H37Rv microorganisms capable of growing well in 25 γ of isonicotinic acid hydrazide or 1-isonicotinyl-2-isopropyl hydrazine per ml. have been obtained after eight to ten transfers. Moreover, in cultures which would be considered sensitive by ordinary methods of titration, relatively resistant variants to each drug may be demonstrated by prolonged incubation in liquid medium or by the inoculation of plates of solid medium containing graded concentrations of the drug.[7]

It is the purpose of the present report to call attention to the fact that isonicotinic acid hydrazide-resistant strains of tubercle bacilli have been recovered relatively early from the sputum of several patients during therapy with these drugs, and to emphasize the significance of this finding.

Six patients with pulmonary tuberculosis were started on isonicotinic acid hydrazide (INAH)[8] or 1-isonicotinyl-2-isopropyl hydrazine (IPH) on January 18, 1952. All had chronic cavitary disease which had not responded satisfactorily to a variety of previous treatments, including streptomycin and para-aminosalicylic acid (PAS). Three patients received INAH orally in dosage of 1 mg. per kg. per day for two weeks, 2 mg. per kg. for two to six weeks, and 4 mg. per kg. thereafter. Three patients were given IPH orally in dosage of 2 mg. per kg. per day for two weeks, followed by 4 mg. per kg. Seventy-two-hour sputum samples were obtained before treatment, and at weekly intervals thereafter. Gaffky counts and cultures were made from the sodium hydroxide-digested and concentrated material. Sensitivity tests were carried out directly from this material by planting equal amounts on tubes of American Trudeau Society egg-yolk-potato-glycerin medium with and without INAH or its isopropyl derivative. For each specimen, a series of 5 tubes were inoculated: 2 control tubes of plain medium, and 3 containing the drug in the concentrations indicated in table 1. With cultures isolated from the sputum of patients

[1] This study was aided (in part) by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service.

[2] ROBITZEK, E. H., AND SELIKOFF, I. J.: Am. Rev. Tuberc., 1952, *65*, 402.

[3] SELIKOFF, I. J., AND ROBITZEK, E. H.: Dis. of Chest, 1952, *21*, 385.

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[5] In subsequent issues of the REVIEW, isonicotinic acid hydrazide will be designated by the newly adopted generic name *isoniazid*. The generic name was decided upon too late for use in this issue.—*The Editors*

[6] STEENKEN, W., JR., AND WOLINSKY, E.: Am. Rev. Tuberc., 1952, *65*, 365.

[7] STEENKEN, W., JR., AND WOLINSKY, E.: Unpublished data.

[8] Supplied as Rimifon and Marsilid, respectively, by Hoffman-La Roche, Inc., Nutley, New Jersey.

TABLE 1

WEEKLY BACTERIOLOGIC STUDIES OF TUBERCLE BACILLI FROM SPUTUM OF SIX PATIENTS WITH CHRONIC CAVITARY PULMONARY TUBERCULOSIS TREATED WITH ISONICOTINIC ACID HYDRAZIDE OR ITS ISOPROPYL DERIVATIVE

PATIENT	DRUG	WEEKS OF THERAPY	GAFFKY†	CULTURE	CONCENTRATION OF DRUG IN γ PER ML. OF SOLID ATS MEDIUM*								
					O‡	O‡	Isonicotinic Acid Hydrazide				1-Isonicotinyl-2-Isopropyl Hydrazine			
							0.125	0.25	0.5	1.0	1.0	2.5	5	10
A	Isonicotinic acid hydrazide	Pre	VII	Postive	4+‖	4+		0	0	0				
		1	VII	Positive	4+	4+		0	0	0				
		2	II	Positive	3+	3+		0	0	0				
		3	III	Positive	2+	3+		2	1	0				
		4	00	Positive	2+	2+		80	6	6				
		5	II	Positive	3+	3+	2+	50	8					
		6	III	Positive	2+	3+	2+	1+	20					
		7	II	Positive	3+	3+	2+	2+	1+					
		8	VI	Positive	3+	3+	3+	2+	2+					
		9	VI	Positive	3+	3+	3+	2+	2+					
		10	VI	—‖										
		11	IX	—										
B	Isonicotinic acid hydrazide	Pre	VI	Positive	4+	4+		0	0	0				
		1	VII	Positive	4+	4+		0	0	0				
		2	VII	Positive	4+	4+		0	0	0				
		3	X	Positive	4+	4+		0	0	0				
		4	VII	Positive	2+	3+		0	0	0				
		5	VI	Positive	3+	3+		0	0	0				
		6	V	Positive	2+	2+	0	0	0					
		7	II	Positive	2+	3+	2	2	0					
		8	III	Positive	2+	3+	2+	1+	1+					
		9	III	Positive	3+	3+	2+	2+	2+					
		10	V	—										
		11	VII	—										
C	Isonicotinic acid hydrazide	Pre	IX	Positive	4+	4+		0	0	0				
		1	IX	Positive	4+	4+		0	0	0				
		2	VII	Positive	3+	3+		0	0	0				
		3	V	Positive	2+	3+		0	0	0				
		4	V	Positive	1+	2+		0	0	0				
		5	IV	Positive	ct¶	ct	3	1	0					
		6	II	Positive	1+	1+	1+	1+	1+					
		7	IV	Positive	2+	3+	3+	2+	2+					
		8	VI	Positive	3+	3+	3+	2+	2+					
		9	VI	Positive	3+	3+	2+	1+	1+					
		10	VII	Positive	3+	3+	3+	3+	2+					
		11	VIII	—										
D**	1-Isonicotinyl-2-isopropyl hydrazine	Pre	IV	Positive	2+	2+					0	0	0	
		1	00	Negative										
		2	00	Negative										
E	1-Isonicotinyl-2-isopropyl hydrazine	Pre	IX	Positive	4+	4+					3+	0	0	
		1	VI	Positive	3+	3+					3+	0	0	
		2	IV	Positive	3+	3+					1+	0	0	
		3	VI	Positive	2+	3+					1+	0	0	
		4	IV	Positive	2+	3+					35	0	0	
		5	III	Positive	2+	2+						1	0	0
		6	III	Positive	1+	2+						ct	5	3
		7	II	Positive	2+	3+						1+	80	30
		8	IV	Positive	2+	3+						2+	2+	35
		9	III	Positive	2+	2+						2+	2+	2+
		10	VI	Positive	3+	3+						3+	3+	2+
		11	X	—										

TABLE 1—*Concluded*

PATIENT	DRUG	WEEKS OF THERAPY	GAFFKY†	CULTURE	CONCENTRATION OF DRUG IN γ PER ML. OF SOLID ATS MEDIUM*								
					O‡	O‡	Isonicotinic Acid Hydrazide				1-Isonicotinyl-2-Isopropyl Hydrazine			
							0.125	0.25	0.5	1.0	1.0	2.5	5	10
F	1-Isonicotinyl-2-isopropyl hydrazine	Pre	00	Negative										
		1	00	Negative										
		2	00	Negative										
		3	00	Negative										
		4	00	Negative										
		5	00	Negative										
		6	00	Positive	40	50						40	20	2
		7	00	Negative										
		8	00	—										
		9	Gastric	Positive										
		10	00	—										

* Concentrations represent total weight of each drug. There is apparently no appreciable loss of drug activity after inspissation.

† For each specimen duplicate slides were examined for ten minutes each. The relative number of acid fast bacilli is indicated as follows:

0 None seen on 2 slides, ten minutes each
I Total of 1–4 bacilli
II Average of 1 bacillus in several fields
III Average of 1 bacillus per field
IV Average of 2–3 bacilli per field
V Average of 4–6 bacilli per field
VI Average of 7–17 bacilli per field
VII Average of 18–25 bacilli per field
VIII Average of 50 bacilli in many fields
IX Average of 100 bacilli in many fields
X Enormous masses on average in many fields

‡ Control tubes without drug.

§ Estimation of the amount of growth made as follows:

4+ Surface of medium covered
3+ Innumerable colonies
2+ Several hundred colonies
1+ 50 to 150 colonies
Actual colony counts if less than 50

‖ Dash indicates culture not finished yet.

¶ Contaminated.

** No positive smears or cultures from end of first week to ninth week.

never treated with these drugs, the minimal concentration necessary for inhibition of growth in such sensitivity tests has been found to be 0.125 γ of INAH or 2.5 γ of IPH per ml.

The weekly Gaffky counts,[9] culture results, and sensitivity tests are shown in the table. It may be seen that definite evidence of increased bacillary resistance to the drugs was noted by the end of the eighth week of treatment in 4 of the 6 patients. In patient A, the beginning of resistance was suggested after twenty-six days, with an increasing amount of growth in the higher concentrations of INAH thereafter.

It will be noted also that the initial fall in the sputum Gaffky counts of these 4 pa-

[9] Although the reliability of the Gaffky count for reporting the number of tubercle bacilli in a sputum specimen is justifiably open to question, it is believed that the reported serial determinations on seventy-two-hour specimens, all carried out with uniform technique by the same experienced technicians, indicate unmistakable trends in the bacillary content.

tients was followed by a progressive rise at a time corresponding closely to the onset of increased bacillary resistance.

Patient F had sputum smears and cultures negative for tubercle bacilli just prior to the start of IPH therapy, despite the presence of pulmonary cavitation. After six weeks of treatment, a positive sputum culture was obtained, and the recovered tubercle bacilli showed a resistance to the drug greater than that of any pretreatment culture so far tested. Tubercle bacilli were again demonstrated in a gastric washing obtained during the ninth week of treatment.

One patient whose sputum was positive before treatment has had negative cultures since one week after therapy was begun. The other 4 patients continued to have sputum positive for *M. tuberculosis* on culture during at least the first nine weeks of treatment.

Subcultures have been made from the strains showing growth in all of the drug-containing tubes; these are being tested in higher concentrations to determine to what ultimate degree they are resistant.

It is well known that the greatest drawback to the use of streptomycin alone in the treatment of chronic pulmonary tuberculosis is the high incidence and early emergence of streptomycin-resistant tubercle bacilli. Early symptomatic and roentgenographic improvement may be followed by relapse to the pretreatment state following the appearance of bacillary resistance. Moreover, patients harboring streptomycin-resistant strains of tubercle bacilli cannot be expected to derive as much benefit from combined chemotherapy with streptomycin and PAS in preparation for necessary surgical procedures as those whose organisms are still sensitive.

The observations reported herein indicate that drug-resistant strains of tubercle bacilli may emerge rapidly in patients with chronic pulmonary cavitary tuberculosis under therapy with INAH or its isopropyl derivative, and that the appearance of such strains may be closely followed by bacteriologic relapse. In the light of these observations it may be predicted that the use of these drugs alone in patients with this type of disease may result in only temporary benefit. Indeed, if bacillary resistance to INAH proves to be as long lasting as it is in the case of streptomycin, and if it is learned from further investigation that the administration of combinations of this series of drugs with other antituberculous agents delays the emergence of drug-resistant strains, then patients harboring INAH- or IPH-resistant strains will be definitely handicapped in so far as their future therapy is concerned. It is important, therefore, that these new drugs not be administered in such a fashion as to encourage tubercle bacilli resistant to their action to appear in large numbers.

It is emphasized that the results described in this report were obtained in patients with chronic cavitary pulmonary tuberculosis. It remains to be seen whether bacillary resistance will appear in other types of disease. These observations, however, indicate the importance of adequate bacteriologic and drug-resistance studies for the evaluation of the hydrazines of isonicotinic acid prior to their general use as therapeutic agents.

In summary: Smears and cultures were made at weekly intervals from seventy-two-hour sputum specimens from 6 patients with chronic cavitary pulmonary tuberculosis under treatment with isonicotinic acid hydrazide or its isopropyl derivative for eleven weeks. Drug-sensitivity tests were done directly from these specimens. The results were as follows: Strains of tubercle bacilli from 4 of the 6 patients showed definite evidence of increasing drug resistance by the eighth week of treatment. The first suggestion of increased resistance was noted in one patient after twenty-six days. These 4 patients showed an initial fall in the Gaffky count followed by a progressive increase in the number of acid-fast bacilli in the sputum occurring shortly after the emergence of the drug-resistant

bacilli. These observations indicate that the isonicotinic acid hydrazide or its isopropyl derivative alone do not constitute adequate therapy for most patients with chronic cavitary tuberculosis. Further investigation should logically proceed to the effect of combinations of the new drugs with other antituberculous agents in order to determine if the emergence of bacillary resistance can be delayed or prevented.

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THE DEVELOPMENT OF DRUG RESISTANCE OF M. TUBERCULOSIS TO ISONICOTINIC ACID HYDRAZIDE[1]

The possibility of the emergence of strains of *M. tuberculosis* with increased resistance towards the antibacterial effect of the new antituberculous hydrazine derivatives appeared to be of considerable practical interest, and has been studied in this laboratory for some time. Although the observations made to the present time are far from complete, it seems justified to report the preliminary findings which indicate that drug resistance of *M. tuberculosis* can be produced to isonicotinic acid hydrazide (INAH) *in vitro*. This result was to be expected, since no chemotherapeutic agent exists to which susceptible strains of organisms may not become resistant under appropriate experimental conditions.

The strain of *M. tuberculosis* H37Rv which is now partially INAH-resistant was obtained by *in vitro* tests in Dubos medium. The usual method of serial transfers was used. It might be mentioned that, by this technique, drug-resistant variants of *M. tuberculosis* H37Rv to 5,000 γ of streptomycin per ml. were obtained in the fifth transfer.

In the present experiments, the first passage with isonicotinic acid hydrazide was carried out by inoculating test tubes containing dilutions of INAH ranging from 100 γ per ml. to 0.0125 γ per ml. with 0.05 ml. of a ten- to fourteen-day-old culture of H37Rv in Dubos medium. The culture of the first tube to show growth after two weeks' incubation (0.033 γ per ml.) was used for the inoculation in the second transfer in an identical series of INAH concentrations, and this manner of transfer was continued and is presently still in progress. There was no significant change in the tuberculostatic concentrations during the first nine transfers. A certain increase of resistance was, however, noted in the tenth to twelfth transfers when INAH concentrations of 0.5 to 1.0 γ per ml. were without bacteriostatic effect. The characteristic break in the sensitivity occurred in the thirteenth passage where a concentration of 25.0 γ per ml. was required to suppress the growth, while 12.5 γ per ml. and lower concentrations were inactive. After that point, drug resistance seemed to proceed at a consistent rate, and in the sixteenth transfer a concentration of 125 γ per ml. was inactive, or a four thousand fold increase of resistance was obtained after 16 transfers. The experiments are being continued.

The partially INAH-resistant strain showed some overlapping resistance toward the chemically closely related 1-isonicotinyl-2-isopropyl hydrazine (IPH). There was a ten- to twenty-fold increase of resistance, the minimal tuberculostatic concentration of IPH being 31 γ per ml.

Information concerning the stability of isonicotinic acid hydrazide resistance and whether the partially resistant strain will also be resistant *in vivo* will be available in the near future.

Thus far, there is no convincing evidence that resistance toward the hydrazine derivatives occurs readily *in vivo*. Twelve strains of H37Rv which were isolated from mice treated with subtherapeutic doses of INAH or IPH (5 to 10 mg. per kg. per day for twenty-one days) showed a completely normal sensitivity to INAH *in vitro;* the growth of all of them was suppressed by a concentration of 0.025 γ per ml. Studies with strains isolated from infected animals after prolonged administration of INAH are now in progress.

The experience of the present writers with freshly isolated strains of *M. tuberculosis*

[1] In subsequent issues of the REVIEW, isonicotinic acid hydrazide will be designated by the newly adopted generic name *isoniazid*. The generic name was decided upon too late for use in this issue.—*The Editors*

from patients undergoing INAH treatment are limited at the present time to six strains originating from patients at Sea View Hospital, Staten Island, New York. This small group includes three from the sputum of patients treated with IPH for two to five months. The sensitivity of all strains toward INAH varied from 0.05 γ per ml. to 0.2 γ per ml., which is considered the normal range of sensitivity. The tuberculostatic concentration of IPH on the six strains was 1.5 γ per ml., i.e., identical with that of the normal strain H37Rv.

Two hundred additional sputum specimens from patients treated with the hydrazine derivatives are now under investigation. The chemotherapeutic sensitivity of all cultures obtained will be determined, and it is hoped that a more complete picture of the possibility of drug resistance can be derived from the forthcoming data. The preliminary nature of the present results makes it impossible to draw any definite conclusions concerning the development of isonicotinic acid hydrazide resistance *in vivo*, even though resistance has been produced *in vitro*.

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IN VITRO STUDIES ON ISONICOTINIC ACID HYDRAZIDE[1]

Isonicotinic acid hydrazide (INAH) has recently been described as having great antituberculous activity *in vivo*.[2] This note presents several aspects of the *in vitro* studies now being carried out with isonicotinic acid hydrazide[3] in our laboratories.

Of particular interest is the great specificity of INAH for *Mycobacteria*. In table 1 is listed the activity of INAH against a varied group of bacteria and fungi; at the levels tested, only a few bacteria and fungi are susceptible to the action of the drug. Only *Mycobacteria* show significant sensitivity.

The methods used were similar to those previously presented[4, 5]. A finer test than the twofold procedure previously described, however, has been used in the present studies. By means of acid-cleaned, sterile 0.2 ml. Kahn pipettes, the following amounts of properly diluted samples are added to 2 ml. volumes of inoculated medium: 0.100, 0.075, 0.057, 0.043, 0.33, 0.025, 0.019, 0.014, and 0.010 ml. After 112 hours of incubation at 37°C., the tests are interpreted before a bright light against a black background. The degree of growth is recorded as (−) for none, (±) for growth less than the controls, and (+) for growth comparable with the controls. The endpoint is considered to be the minimum volume causing complete inhibition. In a (−) (+) series, the (−) is considered the endpoint. In a (−) (±) (+) series, the midpoint between the (−) and (±) tubes is considered the endpoint. Using this method, a representative run of 8 replicates for INAH versus *Mycobacterium tuberculosis* var. *bovis* strain BCG (BCG) gave an average minimal inhibiting concentration (MIC) of 0.0435 γ per ml ± 12.2 per cent (P = 0.95).

INAH was tested against *Mycobacteria* made resistant to other compounds. A culture of BCG was subjected to the action of streptomycin in the above-described test. The MIC was determined and the tests were reincubated for an additional twelve days. At this time the culture was recovered from the endpoint tubes as well as from the tube preceding the endpoint. The culture, after growing out, was re-tested against streptomycin. This was repeated several times to give cultures with stepwise increases in resistance to streptomycin. In table 2 are given the MIC's of streptomycin and INAH against the original strain and two derived strains.

A group of 3 resistant variants of *M. tuberculosis* var. *hominis* strain H37Rv, one resistant to streptomycin, one resistant to *p*-aminosalicylic acid (PAS), and one resistant to both streptomycin and PAS, as well as the original strain, were tested against streptomycin, INAH, and PAS. In table 3 are given the results of this experiment. It is apparent that INAH is as effective against strains of *M. tuberculosis* resistant to streptomycin and PAS as against the original strains.

For studying the development of resistance to isonicotinic acid hydrazide, BCG was subjected to the action of this compound in the manner described above. Two separate experiments were performed, the first being run in duplicate. The results obtained are listed in table 4. It appears that the development of resistance by BCG to INAH *in vitro* is rapid and resembles the one-step mutant caused by streptomycin. Of further interest is the observation that these drug-resistant variants lose their acquired resistance almost

[1] In subsequent issues of the REVIEW, isonicotinic acid hydrazide will be designated by the newly adopted generic name *isoniazid*. The generic name was decided upon too late for use in this issue.—*The Editors*

[2] BERNSTEIN, J., *et al.*: Am. Rev. Tubere., 1952, *65*, 357.

[3] Supplied by E. R. Squibb & Sons as Nydrazid.

[4] RAKE, G., *et al.*: Am. Rev. Tubere., 1949, *60*, 121.

[5] DONOVICK, R., *et al.*: J. Bact., 1950, *59*, 667.

TABLE 1

ANTIBACTERIAL* AND ANTIFUNGAL† SPECTRUM OF ISONICOTINIC ACID HYDRAZIDE

BACTERIA	MIC‡ IN γ PER ML.
Micrococcus pyogenes var. *aureus*	>600
Streptococcus faecalis	>600
Streptococcus pyogenes	>600
Klebsiella pneumoniae	>600
Escherichia coli	>600
Aerobacter aerogenes	>600
Salmonella typhosa	>600
Salmonella schottmülleri	>600
Shigella dysenteriae	>600
Shigella sonnei	>600
Pseudomonas aeruginosa	>600
Proteus vulgaris	>600
Corynebacterium diphtheriae	>600
Diplococcus pneumoniae, Type 2	600
Diplococcus pneumoniae, Type 3	>600
Lactobacillus acidophilus	600
Brucella abortus	>600
Bacillus subtilis	>600
Mycobacterium smegmatis	0.5
Mycobacterium tuberculosis var. *bovis* strain BCG	0.04
Mycobacterium tuberculosis var. *bovis* strain Ravenel	0.06
Mycobacterium tuberculosis var. *hominis* strain H37Rv	0.03
FUNGI	**MIC IN γ PER ML. OF AGAR**
Candida albicans	100
Saccharomyces cerevisiae	100
Ceratostomella ulmi	50
Aspergillus fumigatus	100
Aspergillus niger	100
Penicillium notatum	25
Microsporum audouini	100
Microsporum canis	>100
Epidermophyton floccosum	>100
Cryptococcus neoformans	>100
Trichophyton mentagrophytes, ATCC 9533	100
Trichophyton mentagrophytes, Squibb #1920	>100
Trichophyton tonsurans	>100
Trichophyton rubrum	>100
Trichophyton schoenleini	>100
Trichophyton violaceum	>100
Trichophyton faviforme var. *discoides*	>100
Trichophyton faviforme var. *album*	>100
Trichophyton faviforme var. *ochraceum*	>100

* Twofold dilution procedure in various broths.

† Twofold agar dilution in malt-yeast extracts agar except *Cryptococcus neoformans* which was carried out in broth.

‡ Minimal inhibiting concentration.

as rapidly. After four passages in liquid medium containing no INAH over a period of two months, the strain resistant to 4γ of drug per ml., shown in Experiment II in table 4,

TABLE 2

ACTION OF ISONICOTINIC ACID HYDRAZIDE ON STREPTOMYCIN-RESISTANT STRAINS OF BCG

ORGANISM	MIC* IN γ PER ML.	
	Streptomycin	Isonicotinic Acid Hydrazide
Original BCG	2.17 (6)	0.0618 (8)
BCG after 3 passages against streptomycin	7.96 (4)	0.0464 (4)
BCG after 4 passages against streptomycin	25.9 (6)	0.0278 (8)

* Minimal inhibiting concentration.

Figures in parentheses represent number of assays.

TABLE 3

ACTION OF ISONICOTINIC ACID HYDRAZIDE ON RESISTANT STRAINS OF M. TUBERCULOSIS VAR. HOMINIS STRAIN H37Rv*

CULTURES	MINIMAL INHIBITING CONCENTRATION IN γ PER ML.			
	Isonicotinic Acid Hydrazide	Streptomycin	Para-Aminosalicylic Acid†	
Original H37Rv	0.0284 (8)	1.57 (8)	1,000 – <0.04 (4)	Range of partial inhibition
Streptomycin-resistant variant	0.0368 (8)	>600 (2)	600 – <0.04 (4)	
PAS-resistant variant	0.0223 (8)	1.20 (8)	1,500 (3)	
Both streptomycin- and PAS-resistant variant	0.0112 (2)	>150 (2)	600 (4)	

* Cultures received through the courtesy of W. Steenken, Jr., The Trudeau Foundation, Trudeau, New York.

† These assays run by twofold dilution procedure.

Figures in parentheses represent number of assays.

TABLE 4

THE DEVELOPMENT OF RESISTANCE TO ISONICOTINIC ACID HYDRAZIDE BY BCG

CULTURE	MINIMAL INHIBITING CONCENTRATION IN γ PER ML.		FOLD INCREASE IN RESISTANCE TO ISONICOTINIC ACID HYDRAZIDE
	Isonicotinic Acid Hydrazide	Streptomycin	
Experiment I			
Original culture	0.0451 (10)	—	—
A—After 1 subjection to isonicotinic acid hydrazide	2.68 (4)	—	59
B—After 1 subjection to isonicotinic acid hydrazide	>4.06 (2)	—	>90
Experiment II			
Original culture	0.0458 (6)	0.875 (8)	—
Culture after 1 subjection to isonicotinic acid hydrazide	>4.06 (2)	0.758 (8)	>89

Figures in parentheses represent number of assays.

was again inhibited by approximately 0.04γ per ml. This observation is being investigated more fully. It will be noted that the INAH-resistant strain remains fully sensitive to streptomycin.

The action of a mixture of INAH and streptomycin against BCG was investigated. The mixture was made up, so proportioned that, based on the expected MIC's of streptomycin and INAH versus BCG, the resultant solution was an MIC:MIC proportion. This mixture was tested against BCG in parallel with streptomycin and INAH. The results of two experiments are given in table 5. The data suggest that the action of a mixture of streptomycin and INAH is close to, or slightly less than, additive.

The problems of the effects of mixtures as well as the carbonyl coupling products of INAH on the development of resistance are currently under study. At present it can be said that, after one exposure to the mixture described above, resistance failed to develop

TABLE 5

ACTION OF MIXTURE OF ISONICOTINIC ACID HYDRAZIDE AND STREPTOMYCIN ON BCG

COMPOUND	γ PER ML. PRESENT AT INHIBITION	NUMBER OF MINIMAL INHIBITING CONCENTRATIONS PRESENT AT ENDPOINT	
Experiment I			
Isonicotinic acid hydrazide	0.0718 (5)	1	
Experiment I			
Streptomycin	0.745 (6)	1	
Isonicotinic acid hydrazide, Streptomycin — mixture	Streptomycin 0.541 (7); Isonicotinic acid hydrazide 0.0394 (7)	Streptomycin 0.73; Isonicotinic acid hydrazide 0.55	= 1.28
Isonicotinic acid hydrazide	0.0469 (6)	1	
Experiment II			
Streptomycin	0.917 (6)	1	
Isonicotinic acid hydrazide, Streptomycin — mixture	Streptomycin 0.478 (6); Isonicotinic acid hydrazide 0.0348 (6)	Streptomycin 0.52; Isonicotinic acid hydrazide 0.74	= 1.26

Figures in parentheses represent number of assays.

to either INAH or streptomycin. A more conclusive statement must await continuing studies.

In summary: Isonicotinic acid hydrazide shows high specificity in its action *in vitro* for *Mycobacteria* and poor activity against other bacteria. Moderate activity is displayed against several fungi. Mycobacterial strains resistant to streptomycin and PAS remain sensitive to INAH. Resistance to INAH by BCG develops rapidly *in vitro* and is apparently also lost rapidly. The INAH-resistant strain remains fully sensitive to streptomycin. The action of a mixture of streptomycin and INAH on BCG is close to, or slightly less than, additive.

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STERILIZATION OF TUBERCLE BACILLI BY ISONICOTINIC ACID HYDRAZIDE* AND THE INCIDENCE OF VARIANTS RESISTANT TO THE DRUG IN VITRO

The present report consists of a brief description of some experiments which demonstrate the sterilizing effect of isonicotinic acid hydrazide (INAH) on tubercle bacilli multiplying *in vitro*.

1. Isonicotinic acid hydrazide was added in final concentration of 0.1γ per ml. to a growing culture of the virulent bovine strain, Vallée, in Tween†-albumin liquid medium. At intervals of two, six, and twenty-four hours after initiation of incubation of this culture at 38°C., plate counts were made on oleic-acid–albumin solid agar medium, and parallel studies were carried out on the degrees of acid-fastness of the organisms in this culture. After two and six hours of incubation, plate counts and acid-fastness were the same as in a control tube of the same culture without added INAH. After 24 hours, however, there was a pronounced loss of acid-fastness of more than 95 per cent of the bacterial cells and an approximately twenty-fold drop in the plate count (read after twenty-one days) from the tube containing 0.1γ of INAH per ml. Inasmuch as the great majority of the bacilli of this strain in this medium existed in the form of large and small clumps, these results were of little value for a quantitative estimation of the sterilizing activity of this drug against tubercle bacilli. Nevertheless, it was evident that INAH in relatively low concentration in a short period of time exerts a significant bactericidal effect on this strain of tubercle bacillus.

2. In a similar experiment, a suspension of the R1Rv strain of tubercle bacillus in Tween-albumin growth medium, prepared by a method similar to that described by Fenner,‡ was used. Plate counts from this suspension, which consisted almost exclusively of well-dispersed, single bacterial cells, gave approximately 10^8 colonies per ml. To one 5.0 ml. aliquot of this culture was added 0.5γ of INAH per ml.; to another, 1.5γ of streptomycin per ml.; and to a third, 3.0γ of streptomycin per ml. After twenty-four hours of incubation at 38°C., the tube containing 0.5γ of INAH per ml. yielded approximately 5 by 10^4 colonies per ml.; that containing 1.5γ of streptomycin per ml. yielded 1.5 by 10^8 colonies per ml.; and the tube containing 3.0γ of streptomycin per ml. gave 3 by 10^8 colonies per ml. (plates read after twenty-four days). Thus, at concentrations of INAH which can easily be maintained in the extracellular body fluids of man for at least twenty-four hours,§ this agent is strikingly bactericidal for multiplying tubercle bacilli under these experimental conditions. It should also be mentioned here that, in contrast to a rapid loss of acid-fastness of the bacilli in the culture exposed to INAH, there was no detectable loss of acid-fastness of the bacilli exposed to streptomycin even after several weeks of incubation at 38°C. in these experiments.

It has been found possible by four different methods to detect and isolate variants of tubercle bacilli which are resistant to the antimicrobial action of INAH:

(*1*) Five-tenths of a milliliter of inocula of fully grown (approximately 10^9 living bacterial cells per ml.) Tween-albumin cultures of tubercle bacilli were inoculated into 4.5 ml.

* In subsequent issues of the REVIEW, isonicotinic acid hydrazide will be designated by the newly adopted generic name *isoniazid*. The generic name was decided upon too late for use in this issue.—*The Editors*

† Tween 80 is the proprietary name of the polyoxyethylene derivative of Sorbitan monooleate, obtained from the Atlas Powder Co., Wilmington, Delaware.

‡ FENNER, F., Am. Rev. Tubere., 1951, *64*, 353.

§ ELMENDORF *et al.*, Am. Rev. Tubere., 1952, *65*, 429.

aliquots of oleic-acid–albumin liquid medium containing various concentrations of INAH from 0.02 to 4.0γ per ml. More than 99 per cent of the bacterial cells of the H37Rv, R1Rv, and Vallée strains were inhibited by 0.04 to 0.6γ of INAH per ml. in either Tween-albumin or oleic-acid–albumin liquid medium. The Vallée strain was slightly less susceptible than the other two strains; whereas at concentrations lower than 0.04γ of INAH per ml., all strains grew as well as in media without INAH. After several days of incubation of such cultures (as early as ten days in the case of the Vallée strain, and no later than fourteen days with the other strains), there appeared grossly visible "fluffs" in all concentrations of INAH tested. These fluffs consisted of fully acid-fast masses of serpentine "cords." They continued to grow in the presence of the drug and, after passage in medium without INAH, cultures of these organisms grew equally well in Tween-albumin and oleic-acid–albumin media containing none and 6γ of INAH per ml.

TABLE 1

NUMBERS OF COLONIES OF TUBERCLE BACILLI DEVELOPING IN TUBES OF SEMI-SOLID AGAR MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF ISONICOTINIC ACID HYDRAZIDE AND STREPTOMYCIN

DRUG CONCENTRATIONS PER ML.	IN ISONICOTINIC ACID HYDRAZIDE		IN STREPTOMYCIN	
	R1Rv*	Vallée*	R1Rv*	Vallée*
0	innumerable	innumerable	innumerable	innumerable
0.04	>100	innumerable	—	—
0.06	—	>100	—	—
0.08	30	50	—	—
0.1	30	50	innumerable	innumerable
0.2	30	40	innumerable	>100
0.3	—	—	innumerable	10
0.4	30	25	30	3
0.6	40	25	12	1

* Inocula were approximately 5 by 10^6 bacterial cells per tube.
—Not tested.

(*2*) Five-tenths of a milliliter of inocula of fully grown Tween-albumin cultures of the Vallée and of the R1Rv strains were added to Wassermann tubes containing 2.0 ml. of semi-solid oleic-acid–albumin agar (0.2 per cent). After several days of incubation in the presence of concentrations of INAH from 0.04γ per ml. to 0.06γ per ml. (the highest concentration tested here), discrete colonies of acid-fast "cords" appeared which could be counted at three weeks. The accompanying table shows the results of such an experiment. Analysis of these results shows that there is a higher incidence of variants of these strains resistant to 0.6γ of INAH per ml. than to an equal concentration (by weight) of streptomycin. Also it will be noted that, in contrast to the rapidly decreasing incidence of streptomycin-resistant variants with increasing concentrations of streptomycin, most of the variants which are resistant to relatively low concentrations of INAH are resistant as well to much higher concentrations of this agent.

(*3*) Five-tenths of a milliliter of fully grown Tween-albumin cultures of these same strains of tubercle bacilli were spread on oleic-acid–albumin agar (1.5 per cent) medium containing various concentrations of INAH, on the one hand, and of streptomycin, on the other. Results similar to those obtained with the soft agar cultures were obtained. It is sufficient to record here that the incidence of variants of these strains resistant to

2.0γ of INAH per ml. was of the order of one in 10^7 bacilli, while the incidence of variants resistant to the same concentration of streptomycin was only one in 5 by 10^9. Of some interest is the observation that on oleic-acid–albumin plates containing 0.1 to 0.2γ of INAH per ml., extensive but abnormal growth occurred. The colonies, confluent on most of the surface of these plates, were quite "smooth" and microscopically showed little or no evidence of "cord-formation." Smears from such a plate were stained for acid fastness: it was observed that at least 90 per cent of the bacterial cells were nonacid fast, compared to an incidence of less than 2 per cent nonacid-fast bacilli in smears from colonies on plates containing less than 0.1γ of INAH per ml. This observation is interpreted to indicate that, with such large inocula, some abnormal growth of the tubercle bacilli can occur in these concentrations of INAH, to be followed by partial lysis of many of the bacilli. It should be added that the morphology of the individual resistant colonies which grew out on plates containing up to 2.0γ of INAH per ml. (the highest concentration tested here) was indistinguishable from that of colonies of the parent, susceptible cultures. In these circumstances also it was noted that, in contrast to the rapid decrease in incidence of streptomycin-resistant variants with increasing concentrations of streptomycin, there was a nearly constant incidence of INAH-resistant variants from 0.4 to 2.0γ of INAH per ml.

(*4*) A modification of the gradient plate method described by Szybalski[‖] was employed successfully to compare the incidence of variants of the Vallée and R1Rv strains resistant to INAH and to streptomycin. This technique in the hands of the present writer is not as useful as the plating-out method described above (in (*3*)) for strictly quantitative determinations of the numbers of variants resistant to specific drug levels. Nevertheless, the gradient method permitted demonstration with the greatest ease of the nearly constant incidence of INAH-resistant variants above 0.4γ of INAH per ml. in contrast with the well-known, rapid decline in incidence of streptomycin-resistant variants above 0.4γ of streptomycin per ml.

These INAH-resistant variants of tubercle bacilli were sensitive to streptomycin and, indeed, it has been impossible thus far to detect the emergence of drug-resistant growth of the Vallée strain in tubes of oleic-acid–albumin medium containing both INAH and streptomycin in concentrations as low as 0.1 and 0.2γ per ml., respectively, even after four weeks of incubation at 38°C. This last observation is similar to an earlier demonstration[¶] that the development of selection of variants of tubercle bacilli resistant to chemotherapeutic agents can be strikingly inhibited by simultaneous exposure of these organisms to at least two such drugs whose modes of action differ. It is believed that clinical application of this fact will permit a much greater usefulness of chemotherapeutic measures in tuberculosis and will probably be essential for the public health control of this disease.

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[‖] SZYBALSKI, W., Microbial Genetics Bull., 1951, *5*, 16.
[¶] MIDDLEBROOK, G., AND YEGIAN, D., Am. Rev. Tuberc., 1946, *54*, 553.

BACTERIAL RESISTANCE STUDIES WITH DERIVATIVES OF ISONICOTINIC ACID[1]

Isonicotinic acid hydrazide[2] (INAH) at a concentration of 5γ per ml. is bacteriostatic for the majority of cells of *Mycobacterium ranae*[3] when plated on Difco nutrient agar plus 2 per cent glycerol plus 0.1 per cent yeast extract. Thus, *M. ranae* appears to be twenty times more resistant to INAH than *M. tuberculosis* H37Rv assayed on oleic-acid–albumin agar.[4]

Figure 1 represents the survival curve for a suspension of *M. ranae* plated at increasing concentrations of INAH. It may be clearly seen that, as the majority of the population is gradually inhibited as manifested by a decrease in colony size (broken line in the range of 5 to 10γ per ml.), a resistant fraction of the same population survives up to a five-hundred-fold increase in concentration. The percentage of survivors gradually decreases from approximately 2.4×10^{-2} per cent to about 8×10^{-4} per cent, as the concentration rises from 5 to 100γ of INAH per ml. The growth of resistant colonies becomes slower toward the threshold of complete inhibition at approximately 3,000γ per ml. The majority of resistant colonies appears mucoid in early stages. When the incubation is prolonged beyond a two-week period, new minute colonies arise, increasing the total number of survivors to five times. These minute colonies have not been included in the survival curve or in the derivation of mutation rate.

The curve indicates that in the concentration range of 0 to 3,000γ of INAH per ml., "complete" resistance develops in a single step, but the incidence of survivors resistant to INAH is ten to five hundred times higher than the occurrence of streptomycin-resistant mutants tested in parallel experiments, depending on the increase in concentration of these toxic drugs over the inhibitory level.

The mutation rate to INAH resistance was calculated from the percentage of mutant-free independent cultures.[5] These cultures have been grown in 0.02 ml. of Difco Tb- broth from small inocula of approximately 60 cells, using 0.1 ml. pipettes, and subsequently mixed and incubated in agar plus 500γ of INAH per ml. Preliminary experiments indicate that the collective mutation rate is approximately 1 to 3×10^{-6} per bacterium per generation, thus over 1,000 times higher than in analogous experiments with streptomycin.[6]

First-step resistant colonies vary in their morphology, rate of growth, and susceptibility to INAH. At lower INAH concentrations, the occurrence of intermediary INAH-resistant strains adds to the resemblance between INAH- and streptomycin-patterns of inhibition. Preliminary experiments indicate that during subsequent transfers in the absence of INAH, the resistant strains are overgrown by faster growing sensitive *M. ranae*. Thus, INAH resistance gives the appearance of reversibility under conditions of active bacterial multiplication, but this by no means indicates that resistance will be similarly reversed

[1] This study was aided by a grant from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association.

[2] In subsequent issues of the REVIEW, isonicotinic acid hydrazide will be designated by the newly formed generic name *isoniazid*. The generic name was decided upon too late for use in this issue.—*The Editors*

[3] Obtained through the courtesy of William Steenken, Jr., November 24, 1951, fourth subculture.

[4] STEENKEN, W., JR., AND WOLINSKY, E., Am. Rev. Tuberc., 1952, *65*, 365.

[5] LURIA, S. E., AND DELBRUCK, M., Genetics, 1943, *28*, 491.

[6] HSIE, J., AND BRYSON, V., Am. Rev. Tuberc., 1950, *62*, 286.

in vivo. In the absence of contrary evidence, it is safer to assume that *in vivo* resistance may be practically irreversible.

Figure 1 indicates that first-step resistant colonies do not develop at concentrations over 3 mg. of INAH per ml.; close to this concentration the growth of resistant colonies is strongly inhibited (broken line). By repeated restreaking over a gradient plate,[7] it is possible to obtain growth at levels up to 10 to 20-γ of INAH per ml., but morphology is completely changed and the rate of multiplication strongly impaired. The inhibitory concentration of INAH for other bacteria, for example, *Escherichia coli* and *Micrococcus pyogenes* var. *aureus*, is 10 and 20 mg. of INAH per ml., respectively, thus in the same range as the ultimate upper level for fully resistant *M. ranae*.

1-Isonicotinic-2-isopropyl hydrazine dihydrochloride (IPH) is approximately four hundred times less effective against *M. ranae;* its antituberculous action *in vivo* may depend on the liberation of free INAH, or some other highly potent intermediate compound.

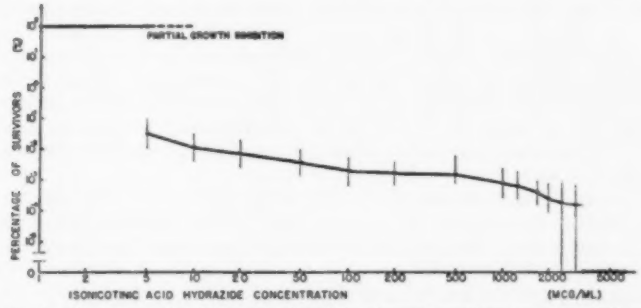

FIG. 1. Survival curve for *M. ranae* plated in glycerol-nutrient agar (10^4–10^9 cells per plate) containing various concentrations of isonicotinic acid hydrazide. The curve represents an average of six independent experiments. Vertical lines indicate the spread of experimental values. In the concentration range 5 to 10 γ per ml., the sensitive population is only partially inhibited (broken line). Consequently, resistant colonies appear on a background of residual growth and are difficult to count. Logarithmic scales are used for both axes.

The shape of the survival curve is quite different for IPH than for INAH: all cells develop colonies up to a level of approximately 2 mg. of IPH per ml. However, gradually impaired growth is noted at concentrations over one-fourth of the inhibitory threshold. The percentage of survivors drops rapidly to zero as the concentration increases from 2 to 2.5 mg. per ml., and single surviving colonies isolated in this range give rise to only slightly more IPH-resistant cultures (less than twofold). These do not show partial inhibition in the range 0.5 to 2 mg. of IPH per ml., and are resistant to INAH. Reciprocally, the INAH-resistant strains of *M. ranae* behave like IPH-"resistant" cultures, exhibiting less than a twofold increase in resistance to IPH.

Cross-resistance studies reveal that the INAH-resistant *M. ranae* do not differ significantly from the parent strain when assayed against aureomycin, bacitracin, chloramphenicol, catenulin, illudin M and S, licheniformin, micrococcin, mycomycetin, neomycin, netropsin, nicotinaldehyde thiosemicarbazone hydrochloride, nisin, *p*-aminosalicylic acid,

[7] SZYBALSKI, W., Bact. Proc., 1952, p. 36.

patulin, polymyxin B and E, penicillin, subtilin, streptomycins, streptothricin, thiolutin, vinactin, and viomycin. Slower growth rate simulates increased sensitivity to certain unstable antibiotics, e.g., terramycin. Reciprocally, strains of *M. ranae* resistant to the above-mentioned drugs do not show major changes in sensitivity when tested against INAH, with the exception of some strains resistant to chloramphenicol, penicillin, netropsin, circulin, polymyxin B and E, licheniformin, and subtilin. The factor of resistance varies from approximately a sixty- to two hundred-fold increase for the first three antibiotics to a two- to thirty-fold increase for the remaining bacterial polypeptides. Among the five chloramphenicol-resistant strains tested, one displays increased sensitivity to INAH.

In combinations with streptomycin, neomycin, or viomycin, the action of INAH is purely additive. In adequate concentration, combinations of these drugs mutually prevent the growth of resistant mutants. However, through successive selection, it is possible to obtain strains resistant to all paired combinations. Similar additive relations exist between INAH-PAS and INAH-nicotinaldehyde thiosemicarbazone, but the high natural resistance of *M. ranae* to these two drugs may make this finding less significant.

In vitro study indicates that with INAH the potential danger of resistance development may be even greater than with streptomycin. However, the first-step resistant mutants of naturally more INAH-susceptible human pathogens may yet prove sensitive to clinically feasible, high levels of INAH. Lack of cross-resistance with other important antituberculous agents suggests the use of multiple chemotherapy to prevent the emergence of resistant strains.

In summary: Isonicotinic acid hydrazide (INAH) shows the streptomycin-like inhibition pattern for *M. ranae*. "Complete" resistance to concentrations exceeding the inhibitory threshold of 5γ of INAH per ml. develops in a single step. The collective mutation rate measured at 500γ of INAH per ml. is approximately 1 to 3 $\times$ 10^{-6} per bacterium per generation, thus over one thousand times higher than for streptomycin.

Absence of practically significant cross-resistance was observed between INAH and 24 other antibacterial agents. In combination with streptomycin, neomycin, viomycin, PAS, or a thiosemicarbazone derivative, the action of INAH was purely additive.

The isopropyl derivative of isonicotinic acid (IPH) has been reported to be highly active *in vivo*, but in the present *in vitro* experiments shows four hundred times lower activity than INAH against *M. ranae*. Significant resistance above this level does not develop, although at high concentrations of the isopropyl derivative, INAH-resistant strains are selected.

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VERNON BRYSON

April 18, 1952

RESISTANCE TO ISONICOTINIC ACID HYDRAZIDE[1]

The development of isonicotinic acid hydrazide as a potentially important antituberculous agent[2–7] has aroused interest in the possible frequency and rate of emergence, from initially sensitive strains, of cells resistant to its action. The present report describes preliminary observations pertaining to this question.

Methods: Crystalline isonicotinic acid hydrazide was used throughout the study. The methods employed in this laboratory in the determination of the sensitivity or resistance of strains of *M. tuberculosis* to drugs have been described in detail elsewhere[8]. In the determination of the population of a given culture, the number of microorganisms per milliliter was determined by the conventional pour-plate procedure, employing the Dubos oleic-acid–albumin solid medium and an incubation period of twenty-one days. Detection of the number of resistant cells among large populations of sensitive cells was facilitated by the use of Szybalski's gradient plate technique[9, 10] and an incubation period of fourteen to twenty days.

Results: The *in vitro* sensitivities of five strains of *M. tuberculosis* to isonicotinic acid hydrazide are illustrated in table 1. Concentrations of 0.05 to 0.4γ per ml. were sufficient for inhibition of growth of these strains when an incubation period of seven days was used. Concentrations ranging from 0.1 to 1.6γ per ml. were required, on the other hand, when an incubation period of twelve days was used. In occasional instances, as indicated in table 1 (strain H37Rv-Bloch) and as further indicated in table 2, a significant change in the apparent sensitivity of a strain occurred after the seventh day of incubation. In some instances, this shift in sensitivity was as great as five hundred- to one thousand-fold. Subcultures of the slow-growing organisms, which appeared in the higher concentrations of isonicotinic acid hydrazide after twelve day's incubation in liquid Tween[11]-albumin medium, onto Szybalski gradient plates containing a maximum concentration of 50γ per ml. indicated that cells resistant to at least 50γ per ml. were present in large numbers. In all instances, the growth was diffuse throughout the entire streak.

The ease with which cells resistant to the action of isonicotinic acid hydrazide could be detected in the above strains suggested the possibility that such cells may be present in unusually high numbers among large populations of sensitive organisms. Subsequent studies have indicated, however, that resistance can be demonstrated in this manner only at times and that the same strains, when tested for their sensitivity to isonicotinic acid hydrazide on repeated occasions, often will show no evidence of cells resistant to this antimicrobial agent.

In a study of 37 strains of human tubercle bacilli, grown on a solid medium containing

[1] In subsequent issues of the REVIEW, isonicotinic acid hydrazide will be designated by the newly formed generic name *isoniazid*. The generic name was decided upon too late for use in this issue.—*The Editors*

[2] BERNSTEIN, J., *et al.*, Am. Rev. Tuberc., 1952, *65*, 357.

[3] STEENKEN, W., JR., AND WOLINSKY, E., Am. Rev. Tuberc., 1952, *65*, 365.

[4] BENSON, W. M., *et al.*, Am. Rev. Tuberc., 1952, *65*, 376.

[5] RUBIN, B., *et al.*, Am. Rev. Tuberc., 1952, *65*, 392.

[6] ROBITZEK, E. H., AND SELIKOFF, I. J., Am. Rev. Tuberc., 1952, *65*, 402.

[7] ELMENDORF, D. F., JR., *et al.*, Am. Rev. Tuberc., 1952, *65*, 429.

[8] HOBBY, G. L., Am. Rev. Tuberc., 1951, *63*, 434.

[9] SZYBALSKI, W., Bact. Proc., 1952, p. 36.

[10] FENNER, F., Am. Rev. Tuberc., 1951, *64*, 353.

[11] Tween 80 is the proprietary name of the polyoxyethylene derivative of Sorbitan monooleate, obtained from the Atlas Powder Co., Wilmington, Delaware.

TABLE 1

THE ANTITUBERCULOUS ACTIVITY OF ISONICOTINIC ACID HYDRAZIDE IN VITRO

STRAIN OF M. TUBERCULOSIS	SENSITIVITY: γ PER ML. (37°C.)		
	Days		
	7	10	12
H37Rv (Bloch)	0.1–0.2	0.1–0.2	0.2–1.6
(Steenken)*	0.1	0.1–0.2	0.1–0.2
Freshly isolated			
Mun. (VMR)†	0.05–0.1	0.1	0.1
Mos.	0.05	0.1	0.1
Bovine-Vallée‡	0.2–0.4	0.2–0.4	0.4

* Streptomycin-resistant mutant received from Wm. Steenken, Jr.

† Viomycin-resistant mutant from freshly isolated strain.

‡ Bovine strain, received from Dr. Cynthia Pierce of the Rockefeller Institute for Medical Research, New York.

Strain "Mos." tested on one occasion only. All other data represent the results of 4 separate tests per culture.

Freshly isolated strains used in this study were obtained through the courtesy of Dr. Ralph Tompsett of the New York Hospital-Cornell Medical Center, and Dr. Daniel Jenkins of Baylor University.

TABLE 2

CHANGES IN THE SENSITIVITY OF STRAINS OF M. TUBERCULOSIS TO ISONICOTINIC ACID HYDRAZIDE AFTER INCUBATION

STRAINS OF M. TUBERCULOSIS	SENSITIVITY: γ PER ML. (37°C.)						
	Liquid Medium						
	Days				Fold Change	Concentrations Subcultured to Solid Media	Results on Solid Medium* 8 Days
	5	7	10	12			
H37Rv							
Bloch	0.1	0.1	0.1	6.3	63.0	3.1	>50
Steenken†	0.05	0.1	25.0	25.0	500.0	12.5	>50
Fresh isolates							
Mun.-VMR‡	0.05	0.05	25.0	50.0	1000.0	25.0	>50
Nit.	0.05	0.1	12.5	25.0	500.0		
Bovine							
Vallée§	0.2	0.2	12.5	50.0	250.0	25.0	>50
Avian							
Kirchberg	25.0	100.0	>100.0	>100.0	>4.0		

* Szybalski gradient plates containing a maximum concentration of 50 γ of isonicotinic acid hydrazide per ml.

† Streptomycin-resistant mutant received from Wm. Steenken, Jr.

‡ Viomycin-resistant mutant from freshly isolated stain.

§ Bovine strain, received from Dr. Cynthia Pierce of the Rockefeller Institute for Medical Research, New York.

Freshly isolated strains used in this study were obtained through the courtesy of Dr. Ralph Tompsett of the New York Hospital-Cornell Medical Center, and Dr. Daniel Jenkins of Baylor University.

varying concentrations of isonicotinic acid hydrazide (i.e., first transfer in the presence of the drug), 9 showed cells resistant to 1γ per ml.; 4, to 10γ; 2, to 50γ; and 2, to 100γ per ml. Insufficient data are available at present to state the actual incidence of such resistant cells among otherwise sensitive populations, but it is of interest that, despite the failure to demonstrate resistant cells in the H37Rv (Block) strain by this method, this same strain, when streaked directly from a liquid medium containing no isonicotinic acid

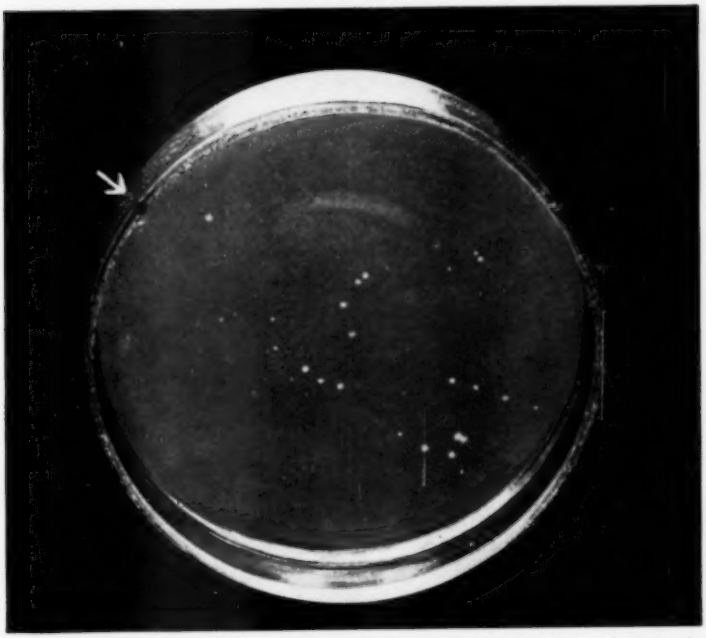

FIG. 1. Gradient plate made according to the method of Szybalski, and containing a maximum of 5 γ (left side of plate) and a minimum of approximately 0.05 γ of isonicotinic acid hydrazide per ml. (right side of plate). The inoculum consisted of 0.1 ml. of a broth culture of H37Rv (Block) which was allowed to flow from point indicated by arrow to opposite side of plate. Arrow indicates also the site of maximum isonicotinic acid hydrazide concentration. Surface colonies indicate presence of cells resistant to concentrations ranging from approximately 0.5 to 4.5 γ per ml.

hydrazide onto a gradient plate containing a maximum concentration of 5γ per ml. (i.e., first transfer in the presence of the drug), showed numerous colonies throughout the streak (figure 1).

In summary: Isonicotinic acid hydrazide is a highly active antituberculous agent *in vitro*. Preliminary observations indicate, however, that cells resistant to its action exist in significant numbers in human strains of tubercle bacilli which have had no previous contact with the drug.

The ease with which these resistant cells have been detected in the experiments reported herein emphasizes the need for careful evaluation of methods currently in use for deter-

mination of the sensitivity of microorganisms recovered from tuberculous individuals, for the laws of chance governing the frequency with which resistance has been detected in these experiments likewise may govern the appearance of resistant cells in primary isolates.

It cannot be predicted at the present time to what extent the existence of resistant cells within freshly isolated strains of *M. tuberculosis* will influence the effectiveness of the drug as an antituberculous agent in man. Their significance will be governed by various factors, including their virulence in man, the rate at which their incidence and/or degree of resistance increases during therapy, and the concentration of isonicotinic acid hydrazide that can be brought into contact with them within the host. It must be assumed, however, that the prevalence of these cells may influence markedly the ultimate value of the compound.

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April 19, 1952

MULTIPLICATION OF TUBERCLE BACILLI WITHIN PHAGOCYTES CULTIVATED IN VITRO, AND EFFECT OF STREPTOMYCIN AND ISONICOTINIC ACID HYDRAZIDE[1]

Problems relating to the mode of activity of chemotherapeutic agents in tuberculosis have recently been discussed in an editorial in *Lancet*, together with a brief report of the results of work done by Mackaness in Sir Howard Florey's laboratory[2] on the activity of some chemotherapeutic agents upon the behavior of tubercle bacilli ingested by macrophages *in vitro*. Similar observations have been made in this laboratory during the past year in the course of a study of the multiplication of tubercle bacilli within mononuclear phagocytes cultivated *in vitro*.

The technique developed for the cultivation and infection of the phagocytes was designed to satisfy the following requirements: (*1*) the phagocytic cells should be cultivated in a single-cell layer in order that they might be readily amenable to microscopic observation; (*2*) the infection of the phagocytes should be minimal at the beginning of the experiment; and (*3*) the proliferation of the tubercle bacilli should take place only within the phagocytes.

Suspensions of mononuclear phagocytes from artificially induced peritoneal exudates in guinea pigs or rabbits were mixed with appropriately diluted suspensions of well-dispersed tubercle bacilli and were then allowed to settle on small coverslips. The cells on the coverslips were then washed, covered with a thin film of Formvar,[3] and cultivated in liquid medium in screw-capped vials. The medium consisted of Hank's solution containing 80 per cent homologous serum, 50γ of penicillin per ml., and various concentrations of the agent to be tested. During the period of cultivation, usually seven days, the monocytes became transformed into macrophages and they spread over the coverslip as a uniform sheet of cells. At the same time the number of tubercle bacilli inside the cells steadily increased from an initial level of 1 to 3 bacilli per phagocyte up to a level of 10 to 50 bacilli per cell on the seventh day.

This technique was used to compare the effects of streptomycin and isonicotinic acid hydrazide on tubercle bacilli growing either in Tween[4]-albumin medium or in cultures of phagocytes. A brief summary of the results is given in the following table:

TUBERCLE BACILLI GROWING IN	MINIMAL CONCENTRATIONS OF DRUG (γ PER ML.) CAUSING COMPLETE INHIBITION	
	Streptomycin	Isonicotinic Acid Hydrazide
Tween-albumin medium	0.5	0.05
Phagocytes *in vitro*	80 to 100	0.05

It is seen that the concentration of streptomycin required to inhibit intracellular growth of tubercle bacilli in phagocytes was approximately one hundred times greater than that required to inhibit extracellular growth in the Tween-albumin medium. By contrast the minimal inhibitory concentration of isonicotinic acid hydrazide was the same for extra-

[1] In subsequent issues of the REVIEW, isonicotinic acid hydrazide will be designated by the newly adopted generic name *isoniazid*. The generic name was decided upon too late for use in this issue.—*The Editors*

[2] Lancet, 1952, *1*, 499.

[3] Formvar, polivynil formal, produced by Shawinigan Products Corp., New York, N. Y.

[4] Tween 80 is the proprietary name of the polyoxyethylene derivative of Sorbitan monooleate, obtained from the Atlas Powder Co., Wilmington, Delaware.

cellular growth in the Tween-albumin medium and for intracellular growth in tissue culture. The results suggest that this type of *in vitro* study may provide additional information on the activity of chemotherapeutic agents *in vivo*.

Using the same technique, intracellular multiplication of tubercle bacilli was studied in relation to virulence and immunity. The results will be published elsewhere.

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(with the technical assistance of Barbara Chalfant)

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April 15, 1952

SYNERGISTIC ACTION OF ISONICOTINIC ACID HYDRAZIDE[1] AND STREPTOMYCIN IN VITRO

Results of many studies on synergistic action between streptomycin and other compounds have been reported in recent years. Experiments showing synergism of isonicotinic acid hydrazide and streptomycin are reported in this note.

Growth-inhibition tests were carried out in Dubos Tween[2]-albumin liquid medium containing various dilutions of the compounds. Two cultures were used, H37Rv (streptomycin sensitive) and H37Rv-R 1,000 (streptomycin resistant). Both are Trudeau Laboratory strains.

TABLE 1

HIGHEST DILUTIONS OF ISONICOTINIC ACID HYDRAZIDE AND STREPTOMYCIN, ALONE OR TOGETHER, WHICH INHIBITED STREPTOMYCIN-SENSITIVE AND STREPTOMYCIN-RESISTANT STRAINS OF MYCOBACTERIUM TUBERCULOSIS

EXPERIMENT	COMPOUND	H37Rv (STREPTOMYCIN-SENSITIVE)	H37Rv (STREPTOMYCIN-RESISTANT)
A	Streptomycin	1:2,000,000	1:200
	Isonicotinic acid hydrazide	1:8,000,000	1:8,000,000
B	Streptomycin	—	1:10,000
	Isonicotinic acid hydrazide	—	1:25,600,000
C	Streptomycin	1:10,000,000	—
	Isonicotinic acid hydrazide	1:16,000,000	—
D	Isonicotinic acid hydrazide	—	1:20,000,000
	Streptomycin	—	1:2,560,000*
E	Isonicotinic acid hydrazide	1,20,000,000	1:20,000,000
	Streptomycin	1:16,000,000	1:2,000,000

* Highest dilution tested.

Tubes containing 5 ml. of medium were inoculated with 0.02 ml. of a ten-day-old culture carried routinely in Dubos medium. Visible growth or no growth was recorded after two weeks' incubation at 37°C. Sterility and growth controls were included in each experiment. The results are shown in the table.

In table 1 (Experiment A) are given the concentrations of isonicotinic acid hydrazide (INAH) or streptomycin required to inhibit growth of the two strains. In Experiment B, the growth of the streptomycin-resistant strain was studied in medium containing a constant, noninhibiting amount of streptomycin (1:10,000 dilution) and decreasing concen-

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[2] Tween 80 is the proprietary name of the polyoxyethylene derivative of Sorbitan monooleate, obtained from the Atlas Powder Co., Wilmington, Delaware.

trations of INAH. In Experiment C, medium containing a constant, noninhibiting amount of streptomycin (1:10,000,000) and decreasing amounts of INAH were inoculated with the streptomycin-sensitive culture. In Experiment D, medium containing a constant, noninhibiting amount of INAH (1:20,000,000) and decreasing concentrations of streptomycin were inoculated with the streptomycin-resistant culture. In Experiment E, carried out separately, medium containing 1:20,000,000 INAH and decreasing amounts of streptomycin were inoculated either with the streptomycin-sensitive or the streptomycin-resistant strain.

The data clearly show synergistic effect with mixtures of these two compounds. Concentrations of streptomycin which are noninhibiting when used alone greatly enhance the growth-inhibiting power of INAH, and vice versa. This effect is shown for both streptomycin-sensitive and streptomycin-resistant strains. It is of particular interest that such effects are obtained with the streptomycin-resistant culture.

These results suggest that combinations of these two agents should be considered for the treatment of human tuberculosis when strains resistant to either agent are found or thought to exist. It is possible that initial concomitant use will make the formation of drug-resistant strains less likely, and that these two drugs can both be used in smaller dosages if used together, rather than if either is used alone.

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April 1, 1952

UREASE ACTIVITY IN MYCOBACTERIACEAE

The identification of bacterial genera and species is for the most part based upon certain biochemical tests which depend on difference of the enzymatic constitution of the organism concerned.

Urease was first found in *Mycobacteria* in 1913 by Soehngen[1] in *M. album* and hyalinum (related to or possibly identical with *M. lacticola*). In 1917, Corper and Sweany[2] demonstrated by very exact methods that strains of human and bovine tubercle possess a ferment capable of splitting urea. The paratubercle bacillus *M. phlei* can grow on simple carbon compounds, deriving its nitrogen from urea or diethylurea.[3] Wohlfeil and Weiland[4, 5] in 1937 stated, however, that strains of *M. tuberculosis* do not contain urease. I. Tsung and S. I. Cheng[6] in 1949 stated that the urease test was of no importance for the identification of *M. tuberculosis*.

As urease production has not been previously recorded in Bergey's Manual, 1948, as a possible characteristic of *Mycobacteriaceae*, the writers have attempted:

(*1*) To devise simple urea media which demonstrate urease activity; (*2*) to study which standard cultures split urea; (*3*) to determine whether it is possible to use urea breakdown for practical diagnostic purposes; (*4*) to study urease activity from a taxonomic point of view as an adjuvant in identifying species or a subdivision of a species.

Methods

A 0.5 per cent urea solution in ammonia-free distilled water was sterilized by filtration through a Seitz filter. One milliliter of the urea solution was heavily inoculated from a Petragnani or Herrold yolk agar slant and tested for ammonia production with Nessler's reagent after two, six, twenty-four hours, or several days. One set of tubes was incubated at 37°C. and the other at room temperature.

Synthetic liquid medium: (1) (without glucose). The medium contained: KH_2PO_4, 1 gm.; K_2HPO_4, 1 gm.; distilled water, 1,000 ml.; phenol red, 12 ml. of a 0.2 per cent solution. It was adjusted to pH 6.8 and after sterilization 20 ml. of a sterile 20 per cent solution of urea were added. The medium was then poured into small tubes in 1 ml. amounts. (*2*) (with glucose). Medium was prepared as above, with the addition of 1 per cent glucose solution. Two sets of tubes were inoculated, one without glucose and one with glucose. Both sets were incubated at 37°C.

Synthetic solid medium: This medium had the same composition as the synthetic liquid medium with the addition of 22 to 25 gm. of washed agar to each 1,000 ml. of medium.

Results

The results of the tests made on the 65 different standard strains of *Mycobacteria* are summarized in table 1.

The strength of reaction and the time required to show evidence of the breakdown of urea by the same culture varied greatly upon repeated testing. The best results were ob-

[1] Soehngen: Centralbl. f.Bakt., 1913, *39*, 8.
[2] Corper, H. J., and Sweany, H. C.: J. Bact., 1917, *3*, 128.
[3] den Dooren de Yong, L. E.: Dissert. Rotterdam, 1926.
[4] Wohlfeil, T., and Weiland, P.: Centralbl. f. Bakt., 1937, *138*, 381.
[5] Wohlfeil, T., and Wohlenberg, H.: Centralbl. f. Bakt., 1937, *140*, 281.
[6] Tsung Cheng, I., and Suei Cheng, I.: J. Formosan med. Ass. Taipeh, 1949, *48*, (9–10), 243.

TABLE 1

RESULTS OF TESTS FOR UREASE ACTIVITY IN 65 STRAINS OF MYCOBACTERIA

MYCOBACTERIUM NAME OF STRAIN	ORIGIN	AMMONIA PRODUCTION IN									
		Urea Solution (Nessler)				Synthetic Liquid				Synthetic Solid	
		2 hrs.	6 hrs.	24 hrs.	Days	2 hrs.	6 hrs.	24 hrs.	Days	24 hrs.	Days
1. 7416 Human H37Rv	NCTC	−	−	+		−	−	−	1	−	±23
2. Human H37Rv	ATCC	−	−	+		−	−	−	4	−	±28
3. 7417 Human H37Ra	NCTC	−	−	−	2	−	−	−	4	−	±26
4. Human (Brevannes)	IPP	−	−	−	2	−	−	−	2	−	±15
5. Bovine (Ravenel)	ATCC	−	−	−	14	−	−	−	5	−	±28
6. Bovine (Dupont)	IPP	−	−	−	5	−	−	−	4	−	±10
7. 6088 Bovine (Cambridge)	NCTC	−	−	−	2	−	−	−	4	−	±8
8. 5693 Bovine (Vallee)	NCTC	−	−	−	3	−	−	−	2	−	±28
9. 7001 Avian WP 1720	NCTC	−	−	−	15	−	−	−	—	−	±18
10. 7092 Avian WP 1745	NCTC	−	−	−	12	−	−	−	10	−	±20
11. Avian (Sheard)	ATCC	−	−	−	12	−	−	−	16	−	±8
12. 946 Cold-blooded type (Friedmann)	NCTC	−	−	−	7	−	−	−	—	−	±3
13. 599 Cold-blooded type	ATCC	−	−	+		−	−	+		+	
14. 9819 Cold-blooded type	ATCC	−	−	+		−	−	+		+	
15. 9823 Cold-blooded type	ATCC	−	−	+		−	−	+		+	
16. B 10*	IRKB	±	+			±	+			−	2
17. Beck*	IRKB	+	+			+	+			−	2
18. Beck RR.*	ISSR	±	+			−	−	+		−	2
19. 280 *Berolinense*	ATCC	−	−	+		−	−	+		+	
20. 356 *Berolinense*	ATCC	−	−	+		−	−	+		+	
21. Boquet*	IPP	+	+			+	+			−	2
22. Brinckerhoff I*	NCTC	±	+			±	+				2
23. Brinckerhoff II*	NCTC	±	+			±	+				2
24. 357 *Butyricum*	ATCC	−	−	+		−	−	+		+	
25. 359 *Butyricum*	ATCC	−	−	+		−	−	+		+	
26. 360 *Butyricum*	ATCC	−	−	+		−	−	+		+	
27. 362 *Butyricum*	ATCC	−	−	+		−	−	+		+	
28. Clegg I*	NCTC	+	+			−	+			+	
29. Clegg II*	NCTC	+	+			−	+			+	
30. Ching Fong*	NCTC	−	−	+		−	−	+		−	2
31. Crottin, Cr 5*	NCTC	+	+			+	−	+		+	
32. Cummins*	IPP	−	−	−	2	−	−	−	2	−	2
33. Currie*	NCTC	+	+			−	+			+	2
34. Danes*	IPP	−	+			−	+			+	
35. Elly*	NCTC	−	−	+		−	−	+		−	2
36. Fish*	NCTC	−	−	+		−	−	+		−	2
37. *Friburgensis**	ICB	−	+			−	−	+		−	2
38. Frog*	ATCC	−	+			−	−	+		−	2
39. Grassberger	IPP	+	+			−	+			−	2
40. Istisan VIII*	ISSR	+	+			+	+			+	
41. Kedrowski*	NCTC	±	+			−	−	+		+	
42. Lanterna*	IPGP	−	−	+		−	−	−	2	−	6

TABLE 1—*Continued*

MYCOBACTERIUM NAME OF STRAIN	ORIGIN	AMMONIA PRODUCTION IN									
		Urea Solution (Nessler)				Synthetic Liquid				Synthetic Solid	
		2 hrs.	6 hrs.	24 hrs.	Days	2 hrs.	6 hrs.	24 hrs.	Days	24 hrs.	Days
43. Mayeranne*	IPP	+	+			+	+			+	
44. *Marinum*	NCTC	−	−	+		−	−	+		−	2
45. Milch*	IRKB	−	+	+		−	+	+		+	
46. Moeller*	IRKB	+	+			+	+			+	
47. Nabarro Bayon*	NCTC	+	+			+	+			+	
48. Needham 361*		−	−	+		−	−	+		−	2
49. 525 *Phlei*—Hay	NCTC	+	+			−	+			+	
50. *Phlei* (Timothy)	ATCC	−	−	+		−	−	+		+	
51. *Ranae*	ATCC	−	−	+		−	−	+		+	
52. 8159 *Smegmatis*	NCTC	−	−	+		−	−	+		+	
53. 10143 *Smegmatis*	ATCC	−	−	+		−	−	+		+	
54. 3820 *Stercoris* 77	NCTC	−	+			−	+			+	
55. 3821 *Stercoris* 281	NCTC	−	+			−	+			+	
56. *Stercoris* 281	ATCC	−	+			−	+			+	
57. Vip 27*	IPP	+	+			+	+			+	
58. Favia*	IIR	−	−	−	4	−	−	−	−	−	4
59. Ninni N16*	IPP	−	−	−	6	−	−	−	−	−	6
60. Butter*	IRKB	−	−	−	10	−	−	−	−	−	15
61. B31*	IPP	−	−	−	14	−	−	−	−	−	16
62. 1472 *Lacticola* 9626	NCTC	−	−	+		−	−	+		+	
63. 9626 *Lacticola*	ATCC	−	−	+		−	−	+		+	
64. 8154 *Lacticola* Pellegrino	NCTC	−	−	−	−	−	−	−	−	−	−
65. Pellegrino*	IPP	−	−	−	−	−	−	−	−	−	−

Blank space means not tested.

— = no ammonia formation.

* = standard strains received from Prof. M. Morellini of the Istituto Carlo Forlanini, Roma.

ATCC—American-Type Culture Collection.

NCTC—National Collection of Type Culture, London.

IPP—Institut Pasteur, Paris.

ISSR—Istituto Superiore di Sanita, Roma.

IRKB—Institut Robert Koch, Berlin.

IIR—Istituto Igiene, Roma.

ICB—Institutul Cantacuzino, Bucuresti.

tained from a fresh subculture grown on Petragnani, Herrold yolk agar, glycerin agar, or washed sediment from Youmans medium.

M. lacticola Pellegrino and *M.* Pellegrino did not show evidence of ammonia formation in synthetic liquid urea medium, and showed very little change of color of the indicator in urea agar medium. When glucose was added to the synthetic liquid urea medium, there was abundant growth but no change in color indicative of the presence of ammonia. Further studies must be carried out in order to investigate the mechanism of the phenomena.

Studies should also be made to determine whether the urease in *Mycobacteria* is an adaptive enzyme or a constitutive one.

From the table it is seen that all of the 65 standard strains (11 true tubercle bacilli, 8 cold-blooded type, 46 paratubercle bacilli), except *M.* Pellegrino, hydrolyzed urea; therefore urease production is a characteristic activity of the genus *Mycobacterium*. On the other hand, 30 strains of paratubercle bacilli hydrolyzed urea in six hours or less, while no strains of true tubercle bacillus produced ammonia in less than twelve hours. It is suggested for practical diagnostic purposes that those strains which show ammonia production in six hours or less be not considered true tubercle bacilli. However, the slow splitting of urea is no proof that the strain concerned is a true *M. tuberculosis*.

In essence, the same method can also be used to differentiate various species of paratubercle bacilli. *Mycobacterium lacticola* Pellegrino and Pellegrino appear not to utilize urea at all; *M.* Favia, Ninni, Butter, and B 31 utilize it rather slowly and *M. lacticola* 9626 quickly. This taxonomic question will be discussed in another publication.

Summary

A simple method is presented for testing urease activity in the family of *Myobacteriaceae*. As 65 standard strains, including 11 true tubercle bacilli, 8 cold-blooded type, 46 paratubercle bacilli, hydrolyzed urea, this is assumed to be a characteristic sign of the family. Urea utilization can be used for diagnostic purposes by eliminating those strains which show ammonia formation in six hours or less as not true tubercle bacilli. Urea breakdown can be used from a taxonomic point of view as an aid in differentiating between species or subdivision of a species.

Acknowledgments

The authors express their gratitude to the National Collection of Type Culture, London; to Professor Moroello Morellini of the Istituto Carlo Forlanini, Roma; and to the Institut Pasteur, Paris, for their kindness and helpfulness in supplying the standard strains.

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January 24, 1952.

THE FAILURE OF CHICK EMBRYO EXTRACT TO ACCELERATE THE GROWTH OF TUBERCLE BACILLI

Introduction

The clumped growth of tubercle bacilli, even in media containing Tween 80 and bovine serum albumin,[1, 2, 3] prevents accurate measurement of growth rate by colony counts. A method was therefore developed for estimating mean generation time in nonturbid cultures by observing the time necessary to reach a given turbidity. The results showed that the bacteria grew more rapidly during the invisible than during the later turbid stages of cultivation.[4] These studies will not be reported in detail because similar results, obtained with an essentially identical method of estimating mean generation time, have meanwhile been published by Youmans and Youmans.[5] The present communication is prompted, however, by inability to confirm the reported accelerating effect of chick embryo extract (CEE) on the growth of tubercle bacilli.[6]

Methods and Materials

The culture media used have been described[1, 2]. The Tween-albumin medium contained mineral salts, 0.2 per cent casein hydrolysate (N-Z Amine, Type B, Sheffield), 0.05 per cent Tween 80, 0.2 per cent glucose, and 0.2 per cent heated serum albumin. The oleic-acid-albumin medium contained mineral salts, 0.2 per cent casein hydrolysate, 0.005 per cent oleic acid, 0.3 per cent glucose, and 0.5 per cent heated serum albumin. Cultures of tubercle bacilli, H37Rv and H37Ra, obtained through the courtesy of Dr. Cynthia Pierce of the Rockefeller Institute for Medical Research, were maintained by weekly transfer of a loopful into 5 ml. of Tween-albumin liquid medium. Chick embryo extract was prepared as described by Bloch,[6] employing fertile eggs obtained from the same source and at the same stage of development (eleven days). For determination of mean generation time, serial tenfold dilutions of 10^{-1} to 10^{-8} ml. of seven-day cultures of H37Rv were inoculated into 5 ml. of Tween-albumin liquid medium in metal-capped 25 mm. test tubes. These were incubated at 37°C. and examined daily for visual evidence of growth.

Results

In various experiments, 10^{-7} inocula of H37Rv in Tween-albumin liquid medium required ten to twelve days to produce visible growth, matching a control tube (stored in the refrigerator) which contained an inoculum of 10^{-1} ml. This multiplication of 10^6-fold represents 20 generations, corresponding to a mean generation time of 12 to 14.4 hours.

In table 1 it is shown that the addition of 0.25 per cent or 1.0 per cent of chick embryo extract did not accelerate growth of H37Rv in Tween-albumin liquid medium; in fact, with the more prolonged incubation required by the smallest inocula, the appearance of growth was delayed by chick embryo extract.

[1] Dubos, R. J., and Davis, B. D., J. Exper. Med., 1946, *83*, 409.
[2] Dubos, R. J., and Middlebrook, G., Am. Rev. Tuberc., 1947, *56*, 334.
[3] Davis, B. D., and Dubos, R. J., J. Exper. Med., 1947, *86*, 215.
[4] Davis, B. D.: Unpublished observations.
[5] Youmans, G. P., and Youmans, A. S., J. Bact., 1949, *58*, 247.
[6] Bloch, H., J. Exper. Med., 1948, *88*, 355.

Chick embryo extract produced a slight turbidity in the cultures which made subsequent observations on growth somewhat difficult. As the experiments proceeded, the bacterial turbidity which developed was relatively granular in the absence of chick embryo extract, and more homogeneous in the cultures containing chick embryo extract. This difference was confirmed by the microscopic finding in cultures without chick embryo extract of serpentine cords typical of H37Rv growing in this medium[7], whereas cultures propagated in the presence of chick embryo extract showed a more dispersed arrangement of the bacilli, with fewer and less dense cords or none at all.

Cultivation of H37Rv in oleic-acid–albumin medium yielded results similar to those described above for Tween-albumin medium. Cultivation of H37Ra in oleic-acid–albumin

TABLE 1

EFFECT OF CHICK EMBRYO EXTRACT ON RATE OF GROWTH OF H37Rv IN TWEEN-ALBUMIN LIQUID MEDIUM

PERIOD OF INCUBATION (DAYS)	INOCULUM (ML.)																							
	10^{-1}			10^{-2}			10^{-3}			10^{-4}			10^{-5}			10^{-6}			10^{-7}				
	Control	0.25% CEE	1.0% CEE	Control	0.25% CEE	1.0% CEE	Control	0.25% CEE	1.0% CEE	Control	0.25% CEE	1.0% CEE	Control	0.25% CEE	1.0% CEE	Control	0.25% CEE	1.0% CEE	Control	0.25% CEE	1.0% CEE			
0	+	+	+																					
1																								
2				+	+	+																		
3							+	+	+															
4																								
5										+	+	+												
6																								
7													+											
8																								
9																+								
10																								
11																			+					
12														+	+									
13																	+	+						
14																				+	+			

medium likewise showed no effect of chick embryo extract on growth rate. In this medium, CEE caused slight dispersion of H37Rv; it had no effect on the already disoriented arrangement of H37Ra, in contradistinction to a previous report.[6]

The mean generation time of H37Rv at invisible population densities observed in these experiments with Tween-albumin media confirms the value (14.4 hours) reported by Youmans and Youmans[5], who used a modified Proskauer and Beck synthetic medium with added 10 per cent beef serum. The failure to confirm (see also[6]) previously reported effects of chick embryo extract on growth rate and dispersion in the same media[6] is not readily explained.

[7] MIDDLEBROOK, G., J. Exper. Med., 1947, *86*, 175.

[8] ROTH, W., Schweiz. Ztschr. f. Path. u. Bakt., 1949, *12*, 451.

Summary

Measurements of growth rate of virulent human-type tubercle bacilli (H37Rv) at invisible population densities have yielded a mean generation time (time for one cell division) of 12 to 14.4 hours in Tween-albumin medium at 37°C.

The reported effect of chick embryo extract on acceleration of growth of H37Rv and H37Ra in Tween-albumin and oleic-acid-albumin liquid media, and on dispersion of bacilli in these media, has not been confirmed.

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American Trudeau Society

Medical Section of the National Tuberculosis Association

Report of the Eighth Pembine Therapy Conference Michigan, Wisconsin, and Minnesota

The eighth Pembine Therapy Conference for pulmonary diseases was held at the Four Seasons Club, Pembine, Wisconsin, September 7, 8, and 9, 1951.

The conference has continued to be an invitational meeting limited to twenty conferees from Michigan, fifteen from Minnesota, and fifteen from Wisconsin, and ten guests from outside the conference area.

Attendance at the conference has been thus limited for the purpose of assuring free discussions of a critical nature.

The conference pattern in recent years has been the presentation of fifty consecutive admissions from a sanatorium in each of the conference states, plus a similar group of cases from some sanatorium from outside the conference area. Tabulation of cases is usually started approximately two years prior to the date of the conference.

In 1950 the conference deviated from the above policy. Forty consecutive admissions from the Thoracic Surgery Services of the University Hospitals of Michigan and Wisconsin and from the Mayo Clinic in Minnesota were presented. Twenty of these were tuberculous cases and twenty were nontuberculous cases.

The committee was of the opinion that this same pattern should be continued for the 1951 conference, with the cases presented from the Veterans Administration hospitals in the three-state area and from one Veterans Administration hospital from outside the area.

The Veterans Administration hospitals at Waukesha and Wood, Wisconsin; Minneapolis, Minnesota; and Dearborn, Michigan presented twenty consecutive tuberculous cases discharged from the tuberculosis services of the above hospitals, and twenty consecutive nontuberculous cases discharged from the nontuberculous chest surgical services of the presentor hospitals. Representative roentgenograms and data prepared by the presentor and submitted to the conferees in table form were reviewed and discussed.

In addition to the above presentations from the conference area, twenty consecutive tuberculous cases discharged from the Veterans Administration Hospital at Sunmount, New York, were presented, following the same pattern as the area hospitals. In addition, the staff of the Sunmount hospital presented for discussion fifty consecutive wedge resection cases.

Summaries compiled in table form containing pertinent data were prepared for each of the conferees. The summaries were prefaced with a discussion of the policies of the presenting hospital, methods of selection of cases, administrative complications, and protocol.

In addition to eight representative roentgenograms, the following outline of data was submitted for each patient:

1. Page number—Case number—Name—Age—Sex—Race
2. Admission date and classification
3. Discharge date and classification
4. History:
    a. Place of previous hospitalization

b. Type of treatment
c. Mode of discovery
d. Date of onset of symptoms until diagnosis

5. Treatment during current period of hospitalization, including dates and complications
6. Significant laboratory data at time of current admission
7. Significant laboratory data following surgery and at the present time, with note as to time of complete conversion of pulmonary secretions in tuberculous cases
8. Present exercise or activity status
9. Total months in sanatorium and hospital
10. In nontuberculous cases, time between diagnosis and surgery
11. Remarks

Following the presentation of cases, each presentor summarized his material. The conference material was submitted in the following order:

I. Friday, September 7, twenty consecutive tuberculous cases discharged from the presentor hospitals in the following order: Wisconsin: Veterans Administration Hospital, Waukesha, Wisconsin, presented by Dr. M. C. Thomas and staff; Michigan: Veterans Administration Hospital, Dearborn, Michigan, presented by Dr. E. G. Nedwicki, Dr. J. C. Day, and Dr. T. L. Jackson; Minnesota: Veterans Administration Hospital, Minneapolis, Minnesota, presented by Dr. W. B. Tucker and Dr. A. Falk; New York: Veterans Administration Hospital, Sunmount, New York, presented by Dr. N. D. D'Esopo, Dr. J. Raleigh, and Dr. B. J. Ryan.

Presentation of the tuberculous cases was completed the first day, allowing two hours for each presentor.

The tuberculous cases were consecutive cases discharged from the tuberculosis services of the above hospitals dating back either from December 31, 1950, or November 30, 1950.

II. Presentation of discharges from the chest surgical services for nontuberculous cases dating back in the above manner.

These cases were presented in the following order: Michigan: Veterans Administration Hospital, Dearborn, Michigan, presented by Dr. T. L. Jackson and Dr. J. C. Day; Minnesota: Veterans Administration Hospital, Minneapolis, Minnesota, presented by Dr. A. Falk, Dr. V. O'Malley, and Dr. W. B. Tucker; Wisconsin: Veterans Administration Hospital, Wood, Wisconsin, presented by Dr. R. Hemphill and Dr. W. Weisel.

Each presentor was again allowed two hours for presentation of his cases.

III. September 9, 1951, presentation of fifty consecutive wedge resection cases by Dr. Nicholas D'Esopo and his staff from the Veterans Administration Hospital, Sunmount, New York.

The following therapy trends were noted in the presentation of the eighty tuberculous cases from the three-state area and from the Veterans Administration Hospital at Sunmount, New York:

1. Pneumothorax was attempted ten times and maintained in four cases.

2. Pneumoperitoneum was initiated and maintained in seventeen cases.
3. There were eighteen thoracoplasties.
4. Fourteen patients had a phreniclasis, most of whom also had pneumoperitoneum. It is to be noted that ten of these phrenics were in Michigan, four in Wisconsin, and none in the other two states.
5. There were eleven resections, ranging from segmentals to pneumonectomies.
6. There were two extrapleural pneumonolyses.

The following observations covering the presentation of this group of eighty cases from the three-state area and from the Veterans Administration Hospital in Sunmount are presented:

1. The use of chemotherapy by the staffs of the presenting hospitals varied considerably. It was pointed out that much of this variance was dictated by protocols used in cooperative investigations.
2. Discharges against medical advice or for disciplinary reasons (with one exception) were more numerous in the presenting Veterans Administration hospitals than in civilian hospitals previously presenting their cases at the conference. The public health hazard inflicted on the home communities by discharges for disciplinary reasons from the Veterans Administration hospitals was emphasized by several of the conferees.
3. In the observations covering the active therapy program there was a definite trend away from pneumothorax, almost to the point of abandoning this procedure in the majority of the hospitals.
4. Pneumoperitoneum with or without phreniclasis received more favorable consideration. However, this procedure also elicited considerable discussion, there being those in the conference who thought that the procedure was of little value and others who presented evidence in favor of its very considerable value, notably Dr. Spencer Schwartz from Oteen, North Carolina, who pointed out that approximately 40 per cent of the patients hospitalized in the Veterans Administration Hospital at Oteen are now being treated with pneumoperitoneum, either as definitive treatment or preparatory treatment.
5. The relative value of dihydrostreptomycin and streptomycin was discussed by Dr. William Tucker. It was pointed out that there is relatively little difference in the therapeutic value of the two drugs, but that deafness is more frequent with the use of dihydrostreptomycin and vestibular changes are more frequent with the use of streptomycin. Dr. Tucker favored the use of streptomycin since the deafness which occasionally results from the dihydrostreptomycin is an irreversible complication.
6. Thirty per cent of the patients were on bed rest alone, apparently without surgical treatment or chemotherapy.
7. The average period of sanatorium care within the presenting hospital was approximately two years. (It should be noted that previous periods of sanatorium care and subsequent periods of hospitalization in other hospitals were recorded in some cases.)

* * *

The presentation of twenty consecutive nontuberculous patients who had major chest surgery from the hospitals above tabulated elicited the following observations and comments:

1. In some instances there appeared to be undue delay in the performance of resectional surgery in questionable cases, some of which later proved to be malig-

nant. This may be one explanation of the fact that, of the fifteen resected carcinomas in the series of sixty cases, twelve are dead within one year. It is recognized, of course, that this very probably is not the only explanation of the high mortality rate in the resected cases.

2. More complete bronchographic examinations prior to resection for bronchiectasis are thought advisable. It was pointed out that the lingula on the left is quite often involved in lower lobe bronchiectasis and that failure to fill this segment may result in leaving an important part of the disease at the time of surgery.
3. In the presentation of the Minnesota series, there was considerable discussion covering the resection of solitary round lesions. There was almost unanimity of opinion that such lesions should be resected. It was pointed out that the presence of calcifications in these lesions does not necessarily rule out the possibility of malignancy.
4. In discussions of the subject of decortication following empyema, it was generally agreed that there is little chance of restoring very much lung function and that this procedure should be reserved for those patients for whom obliteration of the empyema space cannot otherwise be effected.
5. In respect to the treatment of empyema there was considerable discussion concerning the use of Varidase. There was considerable variation in the reported results, but at least some of the reports were encouraging.
6. The problem of persistent bleeding from the entire surgical bed, occurring late during the operative procedure or early postoperative period, received considerable discussion following the presentation of such a case. The possible role of acidosis resulting from carbon dioxide retention in such cases was pointed out. It was further suggested that when the lowered blood pH is corrected, the bleeding might be controlled.

   Nevertheless, the prevention of carbon dioxide retention should receive the attention of the operating team rather than its correction after it has occurred.

* * *

The third morning of the conference was devoted entirely to presentation and discussion of the fifty consecutive wedge resection cases from the Veterans Administration Hospital, Sunmount, New York.

Before and following the presentation of these cases it was pointed out by the presentor and several of the conferees that the report was strictly that of a study to explore the possibilities of long-term streptomycin therapy and excision of residual foci.

The presentor justified these procedures primarily on the premise that many foci which are considered inactive eventually reactivate.

Most of the Sunmount cases had a minimum of four months of daily streptomycin and para-aminosalicylic acid prior to surgery, which was continued for varying periods of time following surgery.

The Sunmount group recommended the excision of lesions greater than 3 mm. in diameter. It was further emphasized that many of these nodules are readily resected in a wedge-shaped manner since most of them are peripheral and present on some pleural surface.

Dr. Edgar Medlar was of the opinion that such foci in the apical and posterior segments of the upper lobe and the superior segment of the lower lobe are the most likely to relapse and that the surgeons at Sunmount are more prone to leave small foci in the anterior division of the upper lobe and in the basal segments of the lower lobe.

Bilateral wedges were resected in twenty-two patients and unilateral procedures in twenty-eight patients. Lobectomy was combined with wedge resection in three patients.

Stages of disease prior to treatment: minimal, 8; moderately advanced, 28; far advanced, 14.

The number of wedges resected per patient varied from one to ten.

All gastric and sputum cultures were negative for tubercle bacilli at the time of resection. In thirty-eight patients, sputum studies are known to be "negative" now; in eleven the results are unknown, and one patient continues to discharge tubercle bacilli.

Dr. Medlar discussed the necrotic character of the resected lesions and presented slides illustrating the pathology of these lesions. He pointed out that, while tubercle bacilli were demonstrated frequently on smear from these foci, cultures failed to reveal growth in many instances. He discussed the problem of the viability of the tubercle bacilli seen in these lesions.

It was again emphasized by the committee and by the presentors that the work at Sunmount as presented is an experimental study.

* * *

The conference was attended by the following guests from outside the conference area other than those from the Veterans Administration Hospital at Sunmount:

Dr. Howard T. Barkley, Texas
Dr. John B. Barnwell, Veterans Administration, Washington, D. C.
Dr. Dumont Clark, Colorado
Dr. John R. Durrance, Veterans Administration, Colorado
Dr. Foster Hampton, Tennessee
Dr. Karl Pfeutze, Illinois
Dr. William S. Schwartz, Veterans Administration, North Carolina
Dr. Henry W. Walters, Veterans Administration, New York
Dr. William W. Woodruff, New York

The committee wishes to express its appreciation for the comments and valued suggestions of the conference guests from other areas of the country.

In Closing, the Chairmen Re-Emphasized the Following Items of Conference Policy:

1. Continued limitation of the number of conferees and guests to sixty persons.
2. Limitation of discussions, in so far as possible, to a critical analysis of the material presented.
3. The following action taken by the conference committee was read to the conference:

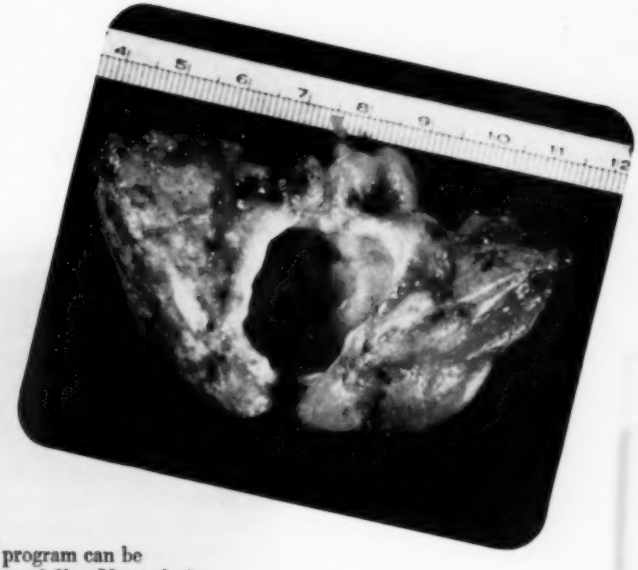
> The Pembine Therapy Conference has given consideration to the desirability of adopting such conference policy as may be necessary to ensure continuity of the purpose for which the conference was established.
>
> At its inception it was the thinking of the founders that the conference should consist of a meeting each year of fifty persons from Minnesota, Wisconsin, and Michigan. It was further contemplated that these fifty persons should be the "principals" from the hospital or area represented. It was our thinking that these men could then return to their "bases" and relay to the associate and junior staff members the concepts and trends of the conference.
>
> From time to time the question has arisen whether or not it might be desirable to have junior staff members and residents present at the conference. It is recognized that this would be desirable in many respects,

but it is also recognized, since this would mean that some of the "principals" could not be invited, that the over-all conference purpose might be jeopardized.

The committee re-affirms the previous position as above outlined and expresses regret that it is not possible to include more junior staff members.

Because of lack of time the area conferees were requested to mail suggestions to the committee.

GEORGE C. OWEN, Wisconsin
EJVIND FENGER, Minnesota
C. J. STRINGER, Michigan



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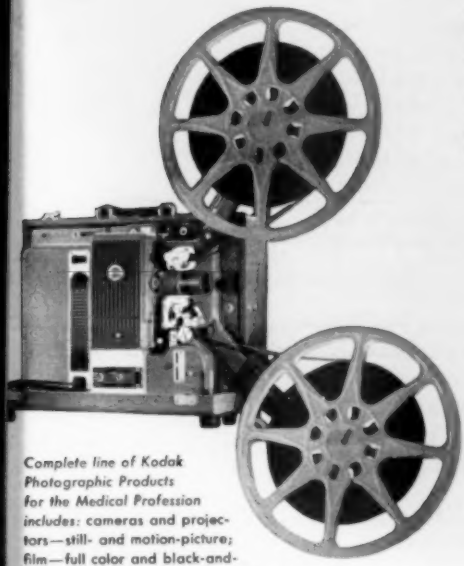

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MEAT... and our changing national caloric requirement

Because of labor-saving devices in present-day occupations, automotive transportation, and fewer hours in the working day, and because population concentrations are moving from rural to urban areas, the resulting changes in living habits have sharply decreased the caloric needs of millions of Americans.[1] Whereas many persons formerly expended 3,500 calories or more daily, today their expenditure may be only 2,500 calories per day. Despite this reduction in caloric requirements, the needs for most essential nutrients—proteins, vitamins, and minerals—remain largely unchanged.

Hence, today more than ever before, foods should be chosen for their high content of essential nutrients in relation to the calories they provide. Foods of high nutritive quality, such as meat, therefore, assume particular importance in the changing American diet.

That meat supplies an abundance of essential nutrients in relation to calories is evident from the table given below. Note that the percentage contribution of the recommended daily dietary allowances made by each nutrient is greater or much greater than the percentage contribution made by the calories.

Calories and Nutrients Provided by 6 oz. of Average Cooked Meat and Their Percentages of Recommended Daily Dietary Allowances

	Amounts per 6 oz. of Average Cooked Meat*	Percentages of Recommended Daily Dietary Allowances N.R.C.†
Calories	454	19%
Protein (biologically complete)	44 Gm.	63%
Iron	5.6 mg.	47%
Phosphorus	4.4 mg.	28%
Niacin	9.5 mg.	79%
Riboflavin	0.44 mg.	24%
Thiamine	0.50 mg.	42%

*Average number of calories and average amounts of the chief nutrients furnished by six-ounce servings of cooked meat (averages of amounts furnished by six ounces each of cooked beef, lamb, pork, and veal).[2]

†National Research Council's recommended daily allowances for a sedentary man (154 lb.).

In addition to the nutrients listed above, meat contributes noteworthy amounts of the vitamins biotin, choline, folic acid, inositol, pantothenic acid, pyridoxine, and the newly discovered vitamin B_{12}.

1. King, C. G.: Trends in the Science of Food and Its Relation to Life and Health, Nutrition Rev. *10*:1 (Jan.) 1952.
2. Watt, B. K., and Merrill, A. L.: Composition of Foods—Raw, Processed, Prepared, in Agriculture Handbook No. 8, United States Department of Agriculture, Bureau of Human Nutrition and Home Economics, 1950.

The Seal of Acceptance denotes that the nutritional statements made in this advertisement are acceptable to the Council on Foods and Nutrition of the American Medical Association.

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